Studies of niacin and acrylamide during processing of instant Asian noodles

A thesis submitted in fulfillment of the requirements
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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

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Publications and presentations

Most of the work reported in this thesis has previously been presented in the following conference papers:

Refereed conference proceedings papers

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Kawila, N., Small, D.M., Marriott, P.J. and Tian, S. (2010) An overview of research on formation of the toxin acrylamide during processing of cereal grain foods, Cereals 2009-Proceedings of the 59th Australian Cereal Chemistry Conference, held from 27st August-30th September 2009, Wagga Wagga, New South Wales, Australia and published by the AACC DownUnder Section, North Ryde, NSW, ISBN 1 876892 37 4, pp16-18.

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Teoh, J.L., Morrison, P.D., Wijaya, M.W., Kawila, N., Sanyoto, C.S. and Small, D.M. (2008) A comparative evaluation of procedures for analysis of ascorbic acid, Cereals 2008-Proceedings of the 58th Australian Cereal Chemistry Conference, held from 31st August-4th September 2008, Surfers Paradise, Gold Coast, Queensland, Australia and published by the AACC DownUnder Section, North Ryde, NSW, ISBN 1 876892 18 1, pp272-273.

Abstract

Instant noodles are a popular food consumed around the world and therefore their significance nutritionally, and to health and wellbeing, are increasingly important issues. Recent research has shown that a number of vitamins are rapidly lost during noodle processing. Accordingly, the first aim of this study has been to extend the previous work to a study of niacin retention in noodles. The second objective was to investigate the possible formation of the toxin acrylamide during noodle processing. The first steps have been to evaluate, adapt and validate analytical procedures for niacin and acrylamide in instant noodles. The resultant methods were then applied to noodle samples particularly to those prepared under controlled conditions in the laboratory.

For instant noodles, niacin has been analysed using a sequential treatment with acid and alkaline solutions followed by solid phase extraction for cleanup of niacin from the complex food matrix, prior to measurement by HPLC with UV detection. The results demonstrated good recoveries as well as symmetrical peak shape and the procedure was found to be suitable for analysis of instant noodle samples. Wheat flour was not a good source of the vitamin so the effectiveness of fortification was evaluated. No significant losses occurred at any of the steps during processing and instant noodles were found to be a suitable vehicle for fortification with no requirement to protect niacin used as a fortificant.

Since the first report of the toxin acrylamide in foods in 2002, there have been various studies on its formation, particularly in potato products. As little is known of acrylamide in Asian instant noodles, this study was designed to firstly establish reliable, sensitive methods for the extraction and analysis of acrylamide for these products. The approach involved derivatisation followed by gas chromatography with electron capture detection in conjunction with the standard addition method as well as use of an internal standard. This procedure has been validated and applied to various samples including commercial instant noodles. Typical levels of acrylamide in the noodles were approximately 60 μg per kg and within the context of the popularity of these products, it is therefore likely

that they contribute significantly to the overall intake of acrylamide for consumers in many countries.

Further investigations have focused on the impact of processing conditions on acrylamide formation in noodles. Temperature and time of frying, as well as pH of the product formulation were all found to significantly influence acrylamide contents. Another approach found to be effective for reducing acrylamide formation is the incorporation of a microbial asparaginase. Suitable dosages of this enzyme and treatment conditions have been investigated and a series of effective strategies are now recommended to minimise the formation of acrylamide during processing of Asian instant noodles.

Table of contents

List of tables

List of figures

Abbreviations

a*	redness (as a component of noodle colour)
AACC	American Association of Cereal Chemists (International)
ABS	Australian Bureau of Statistics
AOAC	Association of Official Analytical Chemists
ASNU	Asparaginase units of enzyme activity
b*	yellowness (as a component of noodle colour)
CW/DVB	Carbowax/divinylbenzene
2,3-DBPA	2,3-dibromopropionamide
ECD	Electron capture detector
FID	Flame ionization detector
GC	Gas chromatography
HPLC	High-performance liquid chromatography
IUPAC	International Union of Pure and Applied Chemistry
L*	lightness value (as a component of noodle colour)
LC	Liquid chromatography
n	the number of replicate analyses used in calculation of individual results
NIST	National Institute of Standards and Technology
NPD	nitrogen-phosphorus detector
$\mathbf{r^2}$	coefficient of determination for a regression curve or line
RDA	recommended dietary allowance
RDI	recommended daily intake
rpm	revolutions per minute
RSD	Relative standard deviations
sd	standard deviation
SPE	Solid phase extraction
SPME	Solid-phase micro extraction
UV	Ultraviolet
WHO	World Health Organization

Explanatory notes

The purpose of these notes is to briefly describe the approaches adopted during the preparation of this thesis. They relate to the units of measurement, spelling, the expression of analytical results, as well as the referencing of literature sources:

1. Where alternative spellings are in common use then the British rather the American approach has been adopted in the text.

2. Generally, for the presentation of results SI units have been used throughout this thesis. Symbols of units used have followed the recent recommendations of the National Institute of Standards and Technology (NIST) (Thompson & Taylor, 2008). In order to facilitate direct comparisons it was necessary to recalculate some values obtained from the literature.

3. Generally experimental data relating to the contents of niacin are presented on a dry weight (or dry matter) basis rather than a fresh weight (or as is) basis unless otherwise clearly specified. The reason that this approach was adopted has been to facilitate direct comparisons of results obtained at different processing stages during manufacture of Asian noodles. It is noted that moisture correction was not required in the presentation of results for acrylamide as all of the samples were tested in the deep fried, dry form. As the moisture contents of all samples were very similar, direct comparisons are possible and can be fairly made.

4. In the citation and listing of references and information sources, the current recommendations of the American Psychological Association (APA, 2001) have been applied throughout. This was chosen on the basis that it is widely adopted and has recently been selected as the format for use in Food Chemistry, one of the leading international journals in the field of Food Science and Technology.

Chapter 1

Introduction

The purpose of this chapter is to provide very brief background and an overview of the research described in this thesis. The context within which this project has been developed includes the following issues:

- Wheat flour and the food products made from that flour are often regarded as good sources of various nutrients that are important to human health and well-being;
- Among these are the B group of vitamins and recent studies have shown that a number of these are subject to significant losses during processing of Asian noodle products. Retention appears to be a problem in each of the three primary types of wheat flour noodles, the traditional white and yellow styles as well as the increasingly popular instant products;
- For some consumers, instant noodles are becoming a staple food due to their convenience and there continues to be a rapid increase in production in many countries well beyond those traditionally known as producers and consumers of noodles. Accordingly these foods are playing an expanding and important role in fulfilling the nutritional requirements of consumers globally;
- Instant noodles can now be considered as potential vehicles for fortification in order to address the issue of ensuring adequate intakes of essential nutrients as well as contributing more generally to health and wellbeing;
- As a vitamin, niacin is also an important dietary component, however there have been no studies of the stability of this nutrient in Asian noodles;
- Whilst wheat flour may be a significant source of vitamins and other nutrients, there is also evidence accumulating in the scientific literature that leads to the hypothesis that the toxin acrylamide is formed during processing of instant noodles;

- Acrylamide is a simple molecule, long utilised in the polymeric form in industrial and laboratory contexts. The monomer is the subject of safety concerns and is classified as “probably carcinogenic to humans”;

- Free acrylamide was first reported in foods in 2002 and since then research has been particularly directed towards potato products which often contain high levels of the toxin. There has been little information regarding acrylamide formation in instant noodles;

- In the context of an interest in estimating dietary intakes of acrylamide, currently there is no standard method to measure this toxin and a need exists for the development of a reliable method that is suitable and has been validated specifically for application to instant noodles; and

- Despite the intensive efforts recently directed towards understanding the significance of acrylamide in foods, the extent of the risks to human health from dietary exposure are still not clear. The emphasis that has been developing from the regulatory perspective and among food processors, involves taking steps to minimise the levels of acrylamide in food products. This has led to the development of a “toolbox” which provides processors with a series of options that can be considered and adapted for a particular food.

Accordingly, the lack of research into niacin and acrylamide in instant Asian noodles, has formed the basis of the project reported in this thesis. The first phases have involved the development of validated procedures which are reliable and effective for analysis of instant noodles. In addition, the stability of niacin during processing of these products has been investigated along with strategies to enhance their nutritional value. Furthermore, the occurrence of acrylamide in a range of commercial products has been surveyed and the factors that influence acrylamide formation in instant noodles have been examined. These studies have been designed so that specific strategies can be recommended for adoption during the processing of instant Asian noodles.

Chapter 2

Background and literature review: The significance, sources and stability of niacin

The purpose of this chapter is to provide background and review the relevant scientific literature on niacin. The areas covered are the chemical structures of the vitamin, the nutritional significance, deficiency symptoms and the adequacy of dietary intakes. In addition vitamin stability under various conditions encountered during food processing and fortification are reviewed.

2.1 B group vitamins as nutrients and food components

Vitamins are divided into two groups on the basis of solubility and these are the water-soluble vitamins and fat-soluble vitamins. The vitamin B group is composed of eight water-soluble vitamins which are thiamin, riboflavin, niacin, vitamin B6, pantothenic acid, vitamin B12, biotin and folates. The B group vitamins are necessary for the metabolism of carbohydrates, fats and proteins and are therefore essential for growth. They are involved in maintaining the health of the hair, skin, nerves, blood cells, immune system, hormone-producing glands and digestive system. As the B vitamins are water soluble, excess to requirements are generally excreted in the urine rather than being stored and a daily intake is necessary to maintain health (Reavley, 1998; Robinson, 1987).

The major dietary sources typically include liver, kidney, whole grains, all seeds, nuts, dairy products, eggs, bran, wheat germ, yeast and green vegetables (Reavley, 1998). Among the B vitamins, four have recently been studied in some detail: the reports have described the analysis of each the vitamins in wheat flour and various styles of Asian noodles, their stability during processing and the potential for fortification of these products. Thiamin (Bui & Small, 2007a, 2007e, 2008b), riboflavin (Bui & Small, 2009), vitamin B6 (Bui & Small, 2008a), as well as the folate group (Bui & Small, 2007b, 2007c, 2007d), including folic acid (Hau Fung Cheung, 2008; Hau Fung Cheung, Hughes, Marriott, & Small, 2009; Hau Fung Cheung, Marriott, & Small, 2007; Hau

Fung Cheung, Morrison, Small & Marriott, 2008) have been studied and in all cases at least 40% of the vitamin content is lost from the formulation during processing and preparation of the noodles. To date there have been no comparable studies of niacin or niacin vitamers and therefore this project has been specifically concerned with the vitamin niacin.

2.2 Chemistry and significance of niacin

Niacin generally refers to nicotinic acid ($C_6H_5O_2N$) and nicotinamide ($C_6H_6ON_2$) and the structures of these are shown in Figure 2.1. It is noted that niacin is similar to a number of the other vitamins, including vitamin C as well as members of the B group in that it is found in foods as vitamers. This term refers to distinct molecular forms of a vitamin each of which has activity as a source of the vitamin nutritionally. In the case of niacin, both nicotinic acid and nicotinamide are utilised by the body, these being the predominant forms of niacin in most foods although there are other molecular forms also present in some food materials.

Niacin has also been referred to as vitamin B3 and the acid and amide forms are interconvertible both chemically and biochemically. In the human body, niacin is used in the production of two important coenzymes in the laboratory and these are nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) (Mark, 1993). In quite recent research it has been found that nicotinic acid can protect against cardiovascular disease and low plasma levels of high-density lipoprotein (HDL) cholesterol (Lebiedzinska & Szefer, 2006).

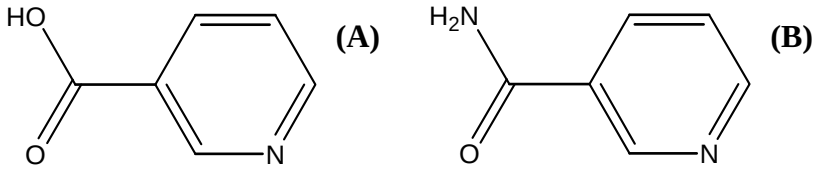


Figure 2.1 The structures of (A) nicotinic acid and (B) nicotinamide

In contrast to the other B group vitamins as well as vitamin C, niacin has been reported to be relatively stable and both molecular forms are quite resistant to heat, light, acid as well as alkaline conditions (Eitenmiller, Landen, & Ye, 2008; Gregory, 2008). The

physical properties of nicotinic acid and nicotinamide are white needle-shaped odourless crystals with molecular weights of 123.11 and 122.12 g/mol, respectively. The solubility of nicotinamide in water is 100 g/100 mL whereas for nicotinic acid it is 1.67 g/100 mL in water (Budavari, 2001) and the pH of a saturated aqueous solution of nicotinic acid is 2.7. The absorption properties of both molecular forms are similar with an absorption maximum near 260 nm and the absorption intensity is not strongly influenced by pH. The molar absorptivity is 2800 per mol per cm at 260 nm in 50mM potassium phosphate buffer at pH 7.0 (Eitenmiller et al., 2008).

2.3 Bioavailability of niacin

The term bioavailability refers to the degree to which an ingested nutrient undergoes intestinal absorption and is therefore able to contribute to metabolic function or be utilised within the body. Bioavailability is considered in the development of the dietary recommendations. In the case of niacin, there are many unavailable forms in many foods and various forms contribute to its incomplete availability in foods of plant origin. The main factor affecting niacin bioavailability is the proportion of the total niacin that is chemically bound (Gregory, 2008). In grains, niacin is present in covalently bound complexes with small peptides and carbohydrates (Combs, 2008).

2.4 Symptoms and effects of niacin deficiencies

Niacin deficiency causes a condition known as Pellagra. This name is derived from the Italian term “pelle agra” for rough skin and the symptoms include sunburn-like rashes on the parts of skin that are exposed to sunlight (face, neck, backs of the hands and forearms) (Combs, 2008) and includes symptoms of dermatosis, dementia and diarrhoea. In addition, niacin deficiency affects various symptoms including insomnia, loss of appetite weight and strength loss, soreness of the tongue and month, indigestion, abdominal pain, burning sensation in parts of the body, vertigo, headache, numbness, nervousness, distractibility, apprehension, mental confusion and forgetfulness. However it was found that pellagra was not an infectious disease but it could be induced by the lack of nutrition associated with corn consumption (Eitenmiller et al., 2008). Niacin deficiency becomes a problem especially if corn and other cereals are major dietary staples.

2.5 Niacin toxicity

The toxicity of niacin is low and non-ruminant animals can tolerate at least 10- to 20-fold excesses to their normal requirements. On the other hand, there are some side effects from the use of high doses in humans, including flushing (an itching urticaria), severe gastrointestinal discomfort (heartburn, nausea, vomitting and rarely diarrhoea), transient increases in the plasma activities of liver enzymes and hyperuricemia (the high level of uric acid in the blood). The last of these results when nicotinic acid in high doses competes with uric acid for excretion causing an increase in the rate of gouty arthritis (Ball, 2004). Niacin toxicity results from the depletion of methyl groups as the result of the metabolism of the vitamin when high doses are taken (Combs, 2008).

2.6 Required levels and adequacy of niacin intakes

The requirements for niacin are related to energy intake because tryptophan is a precursor for the biosynthesis of nicotinic acid and can be converted to niacin in the body. Therefore, nutritionists refer to the number of niacin equivalents (NE) in foods. In humans, approximately 60 mg of dietary tryptophan is considered to produce 1 mg equivalent of niacin. Because a substantial amount of niacin can be synthesised from tryptophan, the nutritional value of niacin is involved with the level of intake of the preformed vitamin but also it includes the potential contribution of the amino acid precursor (Combs, 2008).

The requirements of niacin in the diet have been determined and many countries have established reference or recommended values. These take various forms in different countries. For example, in the United States, the Recommended Dietary Allowances (RDA) values have been defined as “the level of intake of essential nutrients considered in the judgement of the Committee on Dietary Allowances of the Food and Nutrition Board on the basis of available scientific knowledge, to be adequate to meet the known nutrition needs of practically all healthy persons” (Gregory, 2008). In the listing of niacin requirements for human nutrition, use is generally made of niacin equivalents (NE) and these represent the total amount of niacin (the sum of nicotinic acid and nicotinamide, expressed in units of milligrams) plus 1/60 of the amount of tryptophan (also expressed in mg). In the United States, RDA values for niacin range from 27 NE

per day for women up to 41 NE per day for men. In Australia, the "Reference Daily Intake" (RDI) is 16 mg equivalent for men and 14 mg for women.

2.7 Occurrence in foods

Niacin vitamers are found in varying amounts in many foods. Cereal grain foods are not generally regarded as good sources and, as described in Section 2.1, consumption of these may be associated with the development of deficiency symptoms. Selected data for the niacin contents of cereals-based food products are listed in Table 2.1. There is very limited data for niacin contents of Asian noodles and that presented indicates that the levels are relatively low, ranging from 0.2 - 3.0 mg/100g.

It is also well documented that wheat especially the outer (bran) layers of the grain are a good source of the B-group vitamins. Therefore there can be considerable variation in niacin levels in wheat flours depending upon the extraction rates achieved during the milling process. So, for example, flour of 95-100% of extraction rate has been found to contained levels of vitamins higher than that of 40-50% extraction (Cornell & Hoveling, 1998; Gregory, 2008). It is also known that the bioavailability of niacin varies for different foods (Section 2.3) and in plants, it is primarily in the form of nicotinic acid which is protein-bound whereas in animal tissues, nicotinamide predominates and is present as NAD and NADP (Combs, 2008).

2.8 Vitamin fortification of foods

In the early 20th century, a major public health problem that was identified in many countries including the United States was nutrient deficiency. As the various vitamins were discovered and characterised, a corresponding deficiency disease was found. In the case of niacin, the condition was pellagra and this was first reported in the rural south of the United States. Other vitamin deficiencies were also found to be widespread.

Whilst much has been published on the vitamins since those early discoveries, it has been suggested that, even today, in many countries vitamin intakes remain inadequate for at least a part of the population. In addition, vitamin levels in foods are thought to be insufficient to meet the needs of some groups of consumers, even in developed countries (Agostini, Scherer, & Godoy, 2007).

Table 2.1 Food composition table data for niacin contents of cereal-based food products

Description and origin of samples	Niacin content/100g
Pasta made with eggs[a]	1.9 mg nicotinamide
Pasta made with eggs (cooked, drained)[a]	0.4 mg nicotinamide
Wheat (flour) bread (white bread)[a]	0.85 mg nicotinamide
Wheat flour[a]	0.5–5.0 mg
Wheat: Whole grain[b]	3.4-6.5 mg
Wheat: Wheat bran[b]	8.6-33.4 mg
Noodles, egg, raw[c]	2.2 mg
Noodles, egg, boiled[c]	0.2 mg
Pasta, plain, fresh, raw[c]	(0.7) mg
Pasta, plain, fresh, cooked[c]	0.7 mg
Pasta, fresh, cheese and vegetable stuffed, cooked[c]	0.7 mg
Flour, wheat, white, plain[d]	1.9 mg
Noodle, rice stick, boiled[d]	1.9 mg
Noodle, wheat, Asian style[d]	1.0 mg
Noodle, wheat, instant, flavoured, boiled and drained[d]	1.1 mg
Pasta, Egg, boiled[d]	1.4 mg
Pasta, Egg, dry[d]	3.0 mg
Pasta, fresh, white, boiled[d]	1.9 mg
Pasta, white, boiled[d]	1.3 mg
Pasta, white, boiled with salt[d]	1.4 mg
Pasta, white, dry[d]	3.0 mg
Pasta, fresh, white, dry[d]	3.2 mg

Note Sources of data were: [a]Scherz and Senser (2000); [b]Combs (2008);[c]Roe, Finglas and Church (2002); [d]NUTTAB (2006)

The term fortification can be defined as the addition of nutrients in amounts significant enough to ensure that the food is a good to superior source of the added nutrient(s). The term also includes the addition of nutrients not generally associated with the food or addition to levels above that naturally present in the unprocessed food. Legal standards typically provide for the direct addition of several nutrients to foods especially in dairy and cereal-grain products (Gregory, 2008).

Although the fortification of foods with vitamins is widely regarded as potentially beneficial, a series of guidelines have been developed in order to control risks to consumers and to ensure that a reasonable approach is followed. The U.S. Food and Drug Administration guidelines [21CFR Sec. 104.20(g)] state that additions to a food should fulfill the following criteria:

- The nutrient(s) should be stable under conditions of storage, distribution and use;
- They should be physiologically available from the food;
- Excessive intakes will not result.

In Australia, the permission for adding vitamins and minerals to food are provided within the Australia New Zealand Food Standards Code (Standard 1.3.2) which is developed by Food Standards Australia New Zealand. The standard indicates that the fortified vehicle can be either a staple food or a food processed commercially. The general requirements for a food vehicle are that they are commonly consumed, having good stability during storage, of relatively low cost, with no interactions between the fortificant and the food as well as generally involving energy intake (Darnton-Hill & Nalubola, 2002).

2.9 Procedures for analysis of niacin

In the context that the current project seeks to address the identified gap in knowledge about the stability of niacin and potential fortification of Asian instant noodles, the purpose of this section is to review existing knowledge concerning the procedures for analysis of niacin in foods. A wide range of analytical methods have been published and these have involved microbiological, chemical, spectrophotometric as well as fluorimetric approaches. The most commonly applied include the traditional

colorimetric procedure using cyanogen bromide and sulphanilic acid (AACC, 1995b, 1995c; AOAC, 1990b, 1990c), the microbiological assay using *Lactobacillus plantarum* (AACC, 1995d) and, more recently, chromatographic methods.

The classical colorimetric procedure is based upon the König reaction in which nicotinic acid and nicotinamide react with cyanogen bromide to form a pyridinium compound and subsequent rearrangement produces derivative compounds. These couple with aromatic amines to form coloured compounds in the form of a polymethine dye having an absorption peak at 436 nm and the resultant colour is produced in proportion to the niacin content (Eitenmiller et al., 2008). The disadvantages of this method are that it involves the use of hazardous chemicals particularly cyanogen bromide as well as sulphanilic acid. Additionally, the procedure reportedly gives unreliable results, is time consuming and lacks specificity for niacin because all compounds containing a 3-pyridine structure and related derivatives also react (LaCroix & Wolf, 2007).

The standard microbiological assay can also be applied for the determination of niacin using selective microorganisms including *Lactobacillus plantarum, Lactobacillus casei* or *Leuconostoc mesenteroides.* However, this procedure is considered very imprecise, time consuming, labour intensive and is lacking in reproducibility (Lahély, Bergaentzlé, & Hasselmann, 1999).

Various detailed procedures based upon high performance liquid chromatography (HPLC) have been published for the determination of niacin in various foods including meat, coffee as well as fortified foods. The advantages of HPLC are that it permits the separation of nicotinic acid and nicotinamide and it is less time consuming than microbiological procedures (Hidiroglou, Peace, Jee, Leggee, & Kuhnlein, 2008).

2.10 The extraction procedures

Extraction of samples prior to analysis is an important procedure influencing the reliability of results in the estimation of niacin content in foods. Several different approaches are used as the basis for extraction methods that have been published for determining niacin in foods. Typically these use either acid or alkaline solutions or sequential application of both of these. It is considered that only the acid hydrolysable

forms are fully available for humans thus an acid hydrolysis is preferable if the purpose of the analysis is related to determination of the nutritive value of a food (Juraja, Trenerry, Millar, Scheelings, & Buick, 2003). The combined use of acid and alkaline extractants is also utilised for niacin extraction and is thought to release only the bioavailable forms of niacin. Significantly, this approach also has the effect of converting all forms of niacin, including nicotinamide, to nicotinic acid. Among the reagents most commonly used in solutions for extraction are HCl, H_2SO_4, NaOH and $Ca(OH)_2$. The selection of extraction procedures depends upon the following considerations (Eitenmiller et al., 2008):

- Acid treatment can partially convert nicotinamide to nicotinic acid;
- Alkaline treatment can convert nicotinamide to nicotinic acid and this is effectively complete conversion;
- Complete conversion may be advantageous if the objective is to measure the total of the available forms of the various vitamers forms of niacin. This is often used as it simplifies the chromatographic systems required and the analysis, particularly of fortified foods that normally contain both forms.

The terms “total” and “free” (bioavailable) niacin are often used in relation to the niacin components obtained using particular extraction methods and solutions. Total niacin typically refers to the niacin that is extracted by alkaline solutions or by relatively strong mineral acids. The latter includes solutions having a concentration of at least 1N. In contrast, the term free niacin is used to describe that niacin which is extracted by heating or autoclaving with 0.1N mineral acid (Ball, 2006). It has been found that acid extraction with 0.5M H_2SO_4 releases nicotinamide from its coenzyme forms where it is covalently linked to protein components whilst also hydrolysing these to nicotinic acid in cereal products. The alkaline extraction with 0.22M $Ca(OH)_2$ liberates the nicotinic acid from its chemically bound forms and converts nicotinamide to nicotinic acid. However it has been reported that this occurs with a yield lower than 80% (Ball, 2006).

The niacin contents of infant formulations have been determined by autoclaving with 2.5N H_2SO_4 at 121°C for 45 min, followed by SPE (solid phase extraction) using a strong cation-exchange cartridge. The resultant extract was injected into a liquid chromatography (LC) system with an ion-exchange polystyrene-divinylbenzene column

coupled to UV detection at 260 nm. The results obtained were subjected to collaborative evaluation and this provided a peer-verified method for which the results were consistent with certified the niacin values using a suitable reference material (LaCroix & Wolf, 2002). On the other hand, Ndaw, Bergaentzle, Aoude-Werner, and Hasselmann (2002) replaced the acid extraction with enzymatic hydrolysis using a NADase which is able to release the bound forms of niacin to give bioavailable forms. The application of the enzymatic extraction without further use of acid or alkaline hydrolysis was reported to be sufficient to effectively release niacin from foodstuffs containing large quantities of starch and proteins.

2.11 Chromatographic procedures for analysis of niacin

HPLC has been used as the preferred chromatographic approach whereas gas chromatography (GC) techniques have been little reported for analysis of foods due to the low volatility of niacin (Ball, 2006). Capillary electrophoresis (CE) equipped with a UV detector set to read at 254 nm has been used to analyse nicotinic acid in human plasma samples (Zarzycki, Kowalski, Nowakowska, & Lamparczyk, 1995). Similar procedures have been applied for the determination of a wide range of vitamins in pharmaceutical preparations. However, this latter method could not be successfully applied to measurement of the relatively low naturally occurring levels in food as well as beverages. This partially reflects the high levels present in pharmaceutical supplements and indicates the lower sensitivity often associated with CE systems (Trenerry, 2001).

Most published HPLC methods have employed detection of nicotinamide and nicotinic acid using UV absorption at either 261 or 254 nm. Fluorescence properties can also be utilised, although nicotinic acid and nicotinamide do not fluoresce naturally, hence, derivatisation must first be carried out. This can be done using a mixture of cyanogen bromide and *p*-aminoacetophenone and may be carried out as a pre- or post-column step. This technique requires handling of hazardous chemicals, is time consuming and has been reported to give an overestimation of niacin contents (Krishnan, Mahmud, & Matthees, 1999). The derivatisation technique was first applied to human serum by using post-column procedure involving UV irradiation in the presence of hydrogen

peroxide and copper (II) ions and both reagents were added directly to the mobile phase (Mawatari, Iinuma, & Watanabe, 1991).

The investigations by Lahély et al. (1999) established the mechanism of formation of the fluorescent compound from nicotinic acid and nicotinamide by using UV irradiation in the presence of hydrogen peroxide and copper (II) ions. The copper (II) ions catalyse the formation of OH radicals by hydrogen peroxide photolysis and then substitute a hydrogen atom of the pyridine ring into either the *ortho* or *para* position. As a result of the reaction an equilibrium occurs with the corresponding ketone and the fluorescence is monitored by an inline detector. In a more recent study, LC coupled with mass spectrometry (MS) was applied to the determination of niacin in food samples including commercial wheat flour, breakfast cereal and milk samples. An acid extraction followed by SPE with a strong cation exchange column and reversed-phase chromatography showed good accuracy in the analysis of food matrixes (Goldschmidt & Wolf, 2007).

The choice of a suitable chromatographic approach depends upon the extraction and clean-up procedures employed. Some of the alternative modes used in analysis of niacin include strong cation exchange chromatography, reversed-phase ion-pair chromatography, anion exchange chromatography and reversed-phase chromatography. In addition, most of the published methods have used HPLC in conjunction with UV absorbance detection of nicotinic acid and nicotinamide at either 254 or 261 nm (Dawson, Unklesbay, & Hedrick, 1988; Hamano, Mitsuhashi, Aoki, Yamamoto, & Oji, 1988; Hidiroglou et al., 2008; Iwaki, Ogiso, Hayashi, Lin, & Benet, 1994; LaCroix & Wolf, 2001, 2002; LaCroix, Wolf, & Vanderslice, 1999; Van Niekerk, Smit, Strydom, & Armbruster, 1984). However, these wavelengths are not very selective therefore the extraction and clean-up procedures are particularly important. Typically these involve the use of cartridge extractions to effect partial purification and particularly to remove interfering compounds before injecting the solution into the HPLC.

2.12 Clean-up procedures

The determination of niacin is very difficult because of the interfering components of food matrices that co-elute with niacin and absorb at around the wavelength of 260 nm used for detection. Purification has been used to removing interference of UV absorbing

materials. It has been noted that wheat flours have many more endogenous components absorbing at 260 nm than infant formula and these often interfere with the LC analysis when UV detection is employed (Lacroix, Wolf, & Hindsley, 2002).

Various methods to remove these interfering compounds have been described including using a gravity-flow Florisil clean-up column followed by use of an anion-exchange column, column switching or SPE (LaCroix & Wolf, 2002). Van Niekerk et al. (1984) applied the column switching technique to separate the niacin from interfering substances. An open sulfonated Florisil column, washed with 0.5N H_2SO_4 and eluted with 0.5N NaOH is employed to extract niacin in conjunction with monitoring at 260 nm. This method is time-consuming, involving considerable manipulation and requiring a final microfiltration before injection to HPLC (Chase Jr, Landen Jr, Soliman, & Eitenmiller, 1993). Ion-pair chromatography can also improve the resolution of niacin from the interferences and suitable ion-pair reagents include tert-butylammonium hydroxide (TBAH), tetrabutrabutylammonium phosphate (TBAP), sodium dodecylsulfate (SDS), tetrabutylammonium bromide (TBAB) and sodium dioctylsulfosuccinate (Ball, 2006).

2.13 Solid-phase extraction (SPE) techniques

The technique of SPE is applied to isolate vitamins and have been recently used to remove interfering from the complex matrices. This technique is rapid as well as requiring use of less solvents which leads to reduction in pollution as well as presenting a wide sorbent selection (Papadoyannis, Tsioni, & Samanidou, 1997). The time consumed is reduced when compared to liquid-liquid extraction. SPE with the strong cation exchange (SCX) and C18 cartridges has been used to isolate the niacin in food samples including meat, fish and vegetables before injection to an HPLC system and provided a suitable extract which allowed niacin to be well separated from interfering compounds for HPLC analysis (Windahl, Trenerry, & Ward, 1999). LaCroix and Wolf (2002) described the use of SCX-SPE cartridges to extract niacin from infant formula and the niacin extract was recovered by washing with 0.25M NaAc-HOAc buffer at pH 5.6. This method resulted in a complete chromatogram separation when the wavelength of 260 nm was applied for detection. In studies by Iwaki et al. (1994), a Bond Elut SCX

column was used to extract niacin from urine and the recoveries were approximately 80% for nicotinic acid and for some of its metabolites.

2.14 Summary of current knowledge of niacin in foods

In reviewing the literature on niacin analysis, it is clear that the extraction of niacin from food matrices requires removal of interfering materials prior to the final determination. The multiple forms of niacin are referred to the difficulty of extraction techniques. The significance of sample preparation has been investigated for niacin and the application of SPE may also enhance sample preparation. On the basis of the literature reviewed here there is a range of approaches available for analysis of niacin. Although some of these appear useful for the cereal-based food products, the specific challenges of niacin analysis in instant noodles have not been reported and therefore will be evaluated in the current study.

Chapter 3

Background and literature review: Acrylamide

The purpose of this chapter is to provide background and literature review on acrylamide. The areas covered are the chemical structure, toxicology, the occurrence and mechanism of formation as well as an overview of analytical methods.

3.1 Introduction to acrylamide

Acrylamide is a small, relatively reactive molecule and in the monomeric form is primarily used worldwide to synthesize polyacrylamide. Over many years the polymer has been used in the laboratory to separate proteins by electrophoresis (Friedman, 2003). Acrylamide is also used in scientific research to selectively modify SH groups in structural and functional proteins and as a quencher of tryptophan fluorescence in studies designed to elucidate the properties of proteins (Friedman, 2003). Since the 1950s, acrylamide polymers and copolymers have been used in numerous industries including the manufacture of cosmetics and paper, as flocculants during water treatment, as soil conditioning agents and also as grouting agents for dam foundations, tunnels and sewers. Moreover, polyacrylamide is used as a thickening agent in pesticides and also as a medium for hydroponically grown crops.

During railway construction in Sweden, polyacrylamide was used as a grouting agent and some of this sealant entered a nearby stream leading to contamination of the local ground and water systems. The fish and cows died during this construction work and the tunnels workers also showed symptoms of neurotoxin exposure. Further research has been done into the possibility that acrylamide exposure in humans resulted from another source especially cooked foods (Mills, Mottram, & Wedzicha, 2009).

The discovery of acrylamide in foods was first reported in April 2002 by the Swedish National Food Administration and the University of Stockholm. They had observed that acrylamide is formed in a variety of baked and fried foods at high temperature. This announcement attracted worldwide interest because exposure to high levels would be

expected to cause damage to the nervous system. As a result, many researchers have been investigating aspects of the issue including the extent of exposure, health risks, the mechanism of formation of acrylamide as well as strategies for mitigation during food processing.

3.2 Chemical structure and properties of acrylamide

Acrylamide refers to 2-propenamide (C_3H_5NO) which has also been known as, ethylene carboxamide, acrylic acid amide, vinyl amide or propenoic acid amide and the structure is presented in Figure 3.1. Acrylamide appears as a white crystalline solid and the solubilities (expressed in units of g/100mL of solvent at 30ºC) are: water 215.5, methanol 155, ethanol 86.2, acetone 63.1, ethyl acetate 12.6, chloroform 2.66, benzene 0.346, and heptanes 0.0068. The melting point is 84.5°C and its boiling point is 125°C at 25 mmHg and 192.6°C at atmospheric pressure (Budavari, 2001).

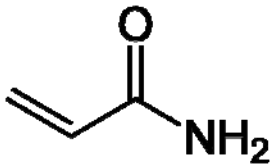


Figure 3.1 The structure of acrylamide

3.3 Toxicology of acrylamide

The International Agency for Research on Cancer has classified acrylamide as "probably carcinogenic to humans" (group 2A) and it is also a known human neurotoxin (Lingnert et al., 2002). The relevant authorities within the European Union have classified acrylamide as a genotoxic carcinogen. Following the first report of acrylamide in foods and as a result of growing concern, the Food and Agriculture Organization of the United Nations and the World Health Organization held a major meeting on the "Health implications of acrylamide in food" within the same year which was 2002 (Taeymans et al., 2004).

Exposure of acrylamide leads to damage to DNA and the nervous system in humans and animals and acrylamide is also considered as a reproductive toxin at high doses. It is known to cause cancer in animals and there is no scientific reason to doubt that similar

effects occur in humans. However, the risk in humans through the intake of foods containing acrylamide cannot be reliably estimated at present (Otles, 2007). Acrylamide is metabolised in the body to glycidamide. The major route of metabolism is then the conjugation of acrylamide and glycidamide to glutathione by a Michael-type reaction leading to water-soluble thioethers (Gökmen & Şenyuva, 2009).

3.4 Occurrence in foods

Acrylamide has been detected in heat-treated foods and is found in carbohydrate-rich matrices that have been subjected to high temperatures during cooking (Mills et al., 2009). In contrast the levels in the raw ingredients are typically difficult to detect. The acrylamide contents reported for various products are presented in Table 3.1. It is clear that acrylamide can be formed in a wide range of foods including products of potatoes, cereal grains, cocoa-based materials as well as beverages. The observations indicate acrylamide formation at high concentration especially in heated-processed foods and also in foods with high temperature of cooking.

Table 3.1 Acrylamide levels in processed foods

Product	Acrylamide (µg/kg)
Potato, Potato crisps	117-4215
Potato, Chips/French fries	59-5200
Potato, Raw	<10-50
Breakfast cereals	<10-1649
Crispbread	<10-2838
Infant food	<10-910
Gingerbread	<10-7834
Diabetics cakes and biscuits	<10-3044
Cookies	25-350
Cakes	43-89
Bread	10-133
Wheat flakes	<10-468
Oat flakes	<10-82
Rice flakes	<10-90
Corn flakes	35-478

Note Sources of data used were Chen, Yuan, Liu, Zhao, and Hu, 2008; Ölmez, Tuncay, Özcan, and Demirel, 2008

Acrylamide intakes have been estimated for populations of a number of countries based on the approach of using dietary records and the results of acrylamide analysis of foods. Daily intake appears to vary globally depending upon local eating habits and cooking routines. Calculated acrylamide intakes range from 0.3 to 0.6 μg/kg body weight per day for adults and 0.4 to 0.6 μg/kg for children and adolescents (Claus, Carle, & Schieber, 2008).

Currently, there are no maximum permitted concentration values for acrylamide in food products within legislative regulations (Kornburst, Stringer, Kerbs, & Hendriksen, 2009). Among western foods, fried and baked potato products, biscuits, bread and coffee account for a large proportion of the dietary exposure. In Germany, bread and bread rolls account for 25% of the acrylamide intake due to the popularity of these foods and a further 20% is attributed to the consumption of home cooked potato chips and similar products (Otles, 2007). However, other food products with low concentration of acrylamide may also contribute substantially to the total acrylamide intakes especially foods that are consumed on a daily basis. It also noted that the acrylamide levels of a variety of foods have not yet been analysed.

3.5 Mechanism of acrylamide formation

Following the discovery of acrylamide in food, researchers identified several mechanisms by which the toxin might form in foods and these potentially involved carbohydrates, proteins, amino acids, lipids as well as other food components. Some of the more likely pathways suggested were:

- Formation via acrolein or acrylic acid; acrylic acid is a possible intermediate to acrylamide and would involve reaction with a source of ammonia;
- Formation via the dehydration/decarboxylation of organic acids such as malic acid, lactic acid and citric acid;
- Direct formation from amino acids via degradation of an α-amino acid initiated by carbonyl compounds.

The last of these three is an example of the group of reactions in foods known as Maillard reactions. These are widely recognised as a type of non-enzymatic browning and have a long history of research in foods since Maillard's initial observations in 1912. In addition to their role in browning reactions they contribute to the development of components responsible for flavour and aroma of foods (Mottram, Low, & Elmore, 2006; Sikorski, Pokorny, & Damodaran, 2008). Therefore, there is already a considerable body of literature and knowledge on the general mechanisms of the Maillard reaction and these may be useful to our understanding of the formation of acrylamide.

Stadler et al. (2002) found that acrylamide can be generated by the thermal treatment of amino acids in the presence of reducing sugars. In that study 20 of the amino acids commonly found in food proteins were individually heated in a model system at 180°C for 30 min. Under these conditions only methionine and asparagine were found to form acrylamide in high concentrations. Heating asparagine and glucose resulted in 368 μmol of acrylamide being formed whilst under the same conditions, methionine formed approximately one-sixth as much. Other amino acids forming low levels of acrylamide included alanine, arginine, aspartic acid, cysteine, glutamine, theonine and valine. Moreover, mass spectral studies confirmed that the three carbon atoms and the single nitrogen of acrylamide originate from the asparagine (Zyzak, et al., 2003). In these early studies the amino acids were added in the free form rather than in the form of bound asparagine which is present in proteins. Subsequent work has confirmed that free asparagine is a key precursor compound in the formation of acrylamide in foods (BeMiller & Huber, 2008). Increasingly evidence has accumulated that asparagine is a key participant in acrylamide formation and the amounts of asparagine in various foods are listed in Table 3.2.

Table 3.2 Levels of free asparagine in food products

Food	Asparagine (mg/kg)
Almonds	980 - 6410
Apples, fresh pulp	315 - 588
Potatoes, fresh	2500 - 3500
Potatoes, dry	580 - 3300
Wheat flour	140 - 190

Note Source of data used was Stadler, 2006

The data in Table 3.2 illustrates that a wide range of asparagine contents is found in a number of common food materials and the highest reported amounts have been found in potatoes. The levels in wheat flour are lower but these may still be sufficient to result in acrylamide formation. This is evidenced from the contents of acrylamide reported in some products prepared from wheat (Table 3.1).

Asparagine is a non-essential amino acid and was first isolated from asparagus juice in 1806 (Friedman, 2003). Asparagine could be a direct precursor of acrylamide formation under pyrolytic conditions. An important reaction is decarboxylation and deamination of amino acids to form a Strecker aldehyde (Mottram, Wedzicha, & Dodson, 2002; Yaylayan, Wnorowski, & Perez Locas, 2003) which is then converted to acrylamide by reaction with a carbohydrate. Potatoes and cereals including barley, rice and wheat contain both of these precursors and this is consistent with the high amounts of acrylamide reported in at least some foods processed using these materials (Table 3.1) and including French fries, potato chips, crisp bread and breakfast cereal products.

The significance of reducing sugars in acrylamide formation from asparagine was first studied by Stadler et al. (2002). Three types of sugars (glucose, fructose and sucrose) were investigated (Table 3.3) and the data indicates that keto sugars such as fructose seem more efficient than aldehydo sugars including glucose. It has been suggested that

this may be due to the lower melting point and greater molecular mobility of fructose (Stadler et al., 2002).

Table 3.3 Acrylamide formation from asparagine and various sugars during heating of a washed whole wheat flour system

Type of sugar	Acrylamide content (μg)
Glucose	72
Fructose	110
Sucrose	92

Note Source of data used was Taeymans et al. (2004)

Following the evidence that free amino acids are precursors of acrylamide, a number of possible pathways have been suggested for its formation in foods. The earliest reports were published by Mottram et al. (2002) and Stadler et al. (2002) and the proposed pathways involved the Strecker reaction of asparagine as the intermediate with subsequent elimination of water at high temperatures resulting in acrylamide formation (Mottram et al., 2002). No evidence for the operation of this pathway has been provided. In addition, Stadler et al. (2002) proposed that the N-glycoside of asparagine is the direct precursor of acrylamide. Subsequent studies have investigated further possible mechanisms.

Zyzak et al. (2003) and Yaylayan et al. (2003) provided evidence to clarify the importance of a Schiff base. Such a molecule is formed by the reaction between asparagine with a carbonyl group of a reducing sugar and according to Zyzak et al. (2003), the decarboxylated Schiff base can react by two possible pathways. The first of these would involve the decarboxylation of the Schiff base to form 3-aminopropionamide, followed by the elimination of ammonia to form acrylamide. In an alternative pathway the decarboxylated Schiff base can directly form acrylamide and the likely mechanisms are presented in Figure 3.2.

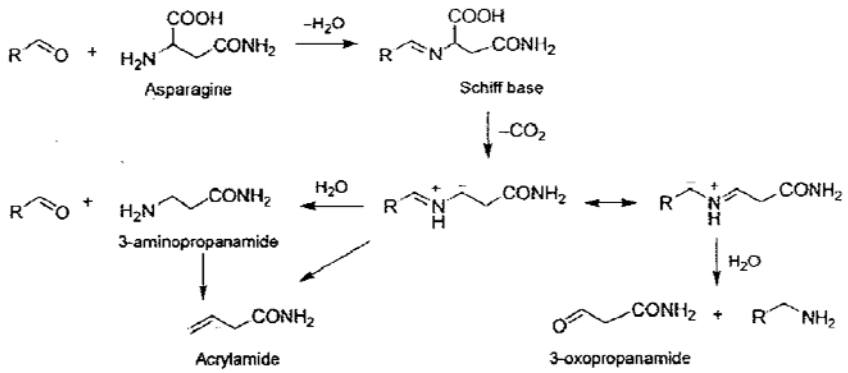


Figure 3.2 The formation of acrylamide via a Schiff base reaction
Note Source of information was Mottram et al. (2006)

Subsequently alternative pathways of acrylamide formation have been described and the mechanisms are summarized in Figure 3.3. Yaylayan et al. (2003) indicated that a Schiff base can rearrange intramolecularly to give an unstable oxazolidine-5-one derivative. This readily decarboxylates to an intermediate azomethine ylide and this readily reacts by an irreversible 1,2-prototropic shift yielding imines 1 and 2. The first of these can hydrolyse yielding 3-aminopropanamide and then eliminate ammonia to form acrylamide. Alternatively, tautomerism of imine 1 can lead to a decarboxylated Amadori compound along with an amino ketone via β-elimination reaction. However, the formation of acrylamide from the Amadori compound is not energetically favoured compared with the azomethine ylide route. Imine 2 cannot produce acrylamide but hydrolysis results in the Strecker aldehyde of asparagine (3-oxopropanamide). An alternative possible pathway is via an Amadori compound. A Schiff base can be rearranged to form an Amadori compound which is also an intermediate for the formation of flavour as well as colour and subsequent β-elimination releases acrylamide. However, Stadler et al. (2002) proposed that the N-glycoside of asparagine is the direct precursor and the potassium salt of N-(D-glucose-1-yl)-L-asparagine is more effective in producing acrylamide than an Amadori compound.

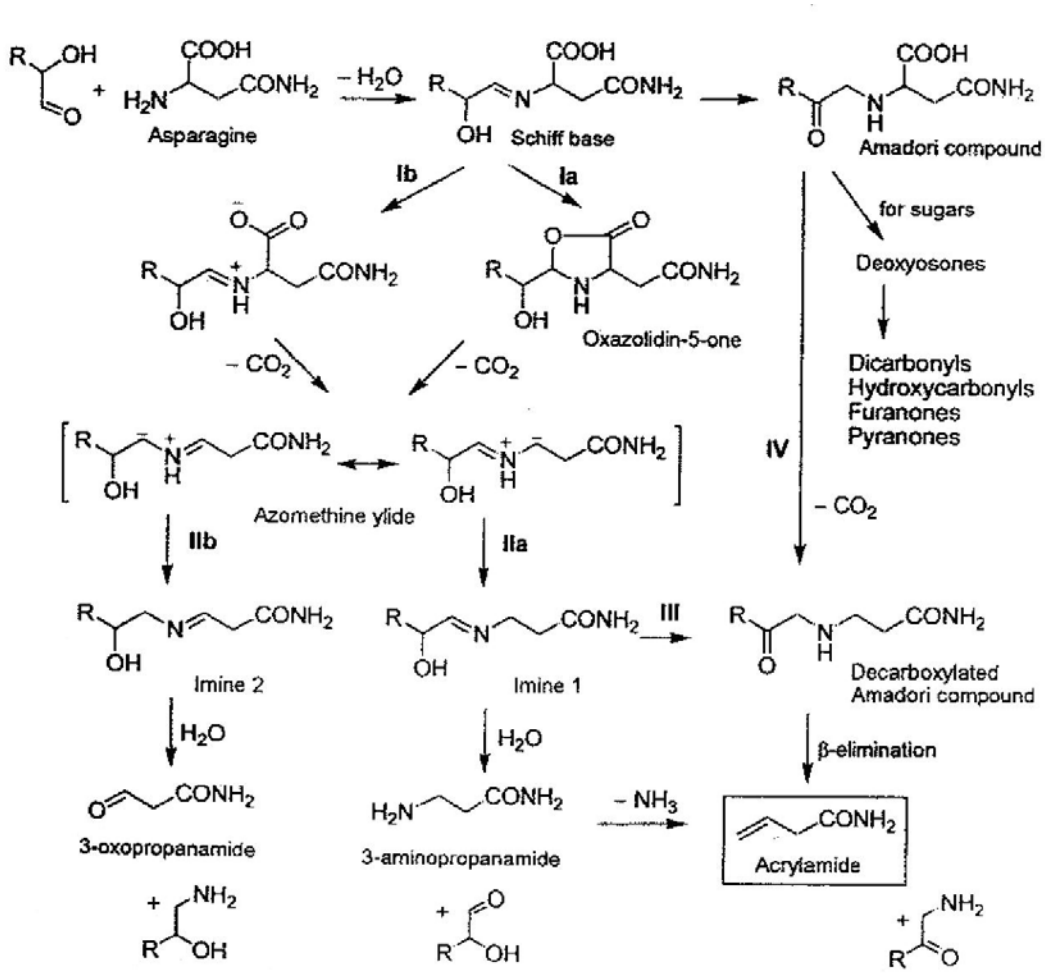


Figure 3.3 The mechanism of acrylamide formation from the reaction of asparagine and reducing sugar

Note Source of information was Mottram et al. (2006)

Although it is now believed that acrylamide in foods is primarily formed through the Maillard reaction, other possible routes have been suggested Gertz and Klostermann (2002) and one of the possible pathways is via acrolein. This has a molecular structure (CH_2=CH-CHO) which is similar to that of acrylamide and is formed during frying at high temperature due to the degradation of lipids (Umano & Shibamoto, 1987). Acrolein is able to react with the ammonia group (NH_3) to form CH_2=CH-CHOH(NH_2) and then oxidation may occur to form acrylamide or reaction with asparagine can form the N-glycoside which is converted to acrylamide (Friedman, 2003). It is known that acrolein can react by oxidation to form acrylic acid and sources of acrolein other than oil include amino acids. Acrylic acid can form acrylamide by reaction with a source of ammonia. It has been concluded that fats are not a significant precursor for acrylamide

formation compared with the established route involving carbohydrate molecules (Mottram et al., 2006).

3.6 Overview of measurement methods

Prior to the discovery of acrylamide in foods, a number of methods had been developed for its determination in other contexts especially in water, biological fluids and non-cooked foods. These methods had been based upon GC or HPLC techniques. Recently, the challenge has to develop reliable and robust methods which allow extraction and measurement in complex food matrices. For this, a relatively high degree of selectivity is typically required and an additional degree of sensitivity is useful because of the complexity of food matrices (Gökmen & Şenyuva, 2009). The first report relating to analysis of acrylamide content in cooked and processed foods was that of Rosén and Hellenäs (2002) who used an isotope dilution LC-MS technique. Since then, various methods have been published and these are primarily based upon MS, coupled with a chromatographic step involving either LC or GC.

The concentration of acrylamide in foods has been found to cover a wide range and this topic has been reviewed in Section 3.4. For highly concentrated products including chips or crisps, acrylamide levels may exceed 3000 µg/kg. Whereas, the acrylamide contents in some foods are quite low. These include products which contribute significantly to intakes because of their high rates of consumption with examples being coffee, infant formula and cereal-based food products with amounts down to 10 µg/kg or lower. Therefore, it is essential that methods with high sensitivity and reliability are available.

3.7 Sample preparation

The determination of acrylamide in complex matrices, including foods having trace levels, requires specific sample extraction and preparation techniques prior to instrumental analysis. The typical steps include sampling, homogenisation, extraction, clean-up and concentration. Another step that is applied in some cases is derivatisation.

A general extraction procedure of acrylamide from foods involves water extraction followed by sample clean-up prior to injection to GC or LC. Potato and cereal-based

food samples are composed of high amounts of colloid-forming components (starch and proteins) as well as high fat contents which can cause erroneous results during analysis. Some published approaches have included a defatting step which is typically carried out with *n*-hexane, petroleum ether or cyclohexane prior to the extraction step (Biedermann et al., 2002; Dunovská, Cajka, Hajslová, & Holadová, 2006; Hoenicke, Gatermann, Harder, & Hartig, 2004; Mastovska & Lehotay, 2006). A further challenge for some foods is that high protein samples require the removal of protein by adding methanol, acetronitrile or saline solution. Alternatively, the addition of Carrez reagents has been reported (Delatour, Périsset, Goldmann, Riediker, & Stadler, 2004). This entailed the use of 1 mL of 0.68M potassium hexacyanoferate (II) trihydrate (Carrez I) and 1 mL of 2M zinc sulfate heptahydrate solution (Carrez II) and these were added while swirling for chocolate powder, cocoa as well as coffee samples. Protein was observed to form a precipitate and accurate analytical results were reported using this approach. In addition, Gertz and Klostermann (2002) found that Carrez solution effectively removed interfering substances in fatty samples including crisps. For matrices high in fat, this material was separated by filtration of the clarified solution because unsaturated fatty acids interfered in subsequent stages of the analysis.

Water has been used to extract acrylamide at room temperature in most of the analytical procedures reflecting the hydrophilic nature of the molecule (Becalski, Lau, Lewis, & Seaman, 2002; Rosén & Hellenäs, 2002; Tareke, Rydberg, Karlsson, Eriksson, & Törnqvist, 2000; 2002). Apart from water, methanol can be considered as an alternative extractant because it also readily solubilises acrylamide (Gökmen & Şenyuva, 2006a; Gökmen, Şenyuva, Acar, & SarIoglu, 2005; Tateo & Bononi, 2003). Other polar solvents including acetronitrile, propanol, ethanol/dichloromethane mixture as well as mixtures of water and organic solvents particularly *n*-propanol or 2-butanone have been also used to extract acrylamide from selected foods (Biedermann et al., 2002). Water at higher temperatures (60-80°C) has been used to facilitate extraction although it also provided satisfactory results when used at room temperature (Castle, 2006). In some studies, cereal and potato products have been extracted at room temperature while fatty matrices, such as those found in peanut butter and chocolate, were extracted at high temperature. Extraction performed after a defatting step was found to be more effective because swelling properties of the remaining matrix changed when fat was removed (Hoenicke et al., 2004).

On the other hand, Gökman and Şenyura (2009) suggested that, although water and methanol can be used for extraction, problems may arise because other components including starch, proteins and sugars are soluble in water and methanol. Therefore, a single-stage procedure may give a poor yield and to solve this problem multiple-stage approaches might be used to increase extraction rate. For potato chips, Soxhlet extraction with methanol enhanced extraction nearly seven fold compared to water extraction (Pedersen & Olsson, 2003).

3.8 Derivatisation of sample extracts

In order to facilitate instrumental analysis derivatisation can be incorporated into the preparative procedure. Acrylamide can be readily brominated to give 2-bromopropenamide (2-BPA) and the overall reaction is presented in Figure 3.4. It is known that 2,3-dibromopropionamide (2,3-DBPA) also forms but this is converted to 2-BPA. These derivatives are far less polar compared to acrylamide and so are readily soluble in non-polar organic solvents including ethyl acetate and hexane.

CH_2=CH-CO-NH_2 + Br_2 $\rightarrow$ CH_2=CBr-CO-NH_2

Figure 3.4 The overall bromination reaction of acrylamide

It has been reported that for GC separation, derivatisation of acrylamide is required prior to injection into the instrument (Zhang, Dong, Ren, & Zhang, 2006) and this can be done with hydrobromic acid, potassium bromide or a saturated bromine-water solution. The excess bromine is removed by addition of sufficient sodium thiosulfate so that the solution becomes colourless. The advantages of bromination of acrylamide to 2,3-DBPA are improved selectivity, increased volatility, removal of potentially interfering co-extractives, giving excellent GC properties and providing a good response both for the electron capture detector (ECD) as well as the nitrogen-phosphorus detector (NPD). Moreover, higher sensitivity is achieved and another advantage is that no further clean up is required after bromination (Taeymans et al., 2004).

3.9 Solid-phase microextraction (SPME)

In order to achieve a practical and reliable method for the analysis of complex matrices, a series of strategies have been investigated and these including treatment of sample extracts. Typically, more than 80% of the analysis time is spent on sample preparation and so the minimisation of preparation steps has been explored and SPME has been suggested as a possible choice.

SPME was originally developed by Arthur and Pawliszyn (1990) as a pre-concentration technique for the analysis of water pollutants. Rather than using non-polar solvents for extraction, SPME uses a fused-silica fiber that is coated on the outside with a relatively thin film of several polymeric stationary phases. This film is selected so that it attracts the molecule of interest and concentrates these on the surface during absorption or adsorption from the sample matrix. The analyte in the sample is directly extracted and concentrated to the fiber coating. The approach has been particularly useful when used in combination with GC and can be applied to a wide range of samples. Lee, Chang, and Dou (2007) reported that direct immersion SPME coupled to GC-MS provided a precise method for analyzing trace amounts of acrylamide from aqueous samples and this analytical procedure was applied to the quantification of the levels in French fries and potato chips.

A range of specific fibres with various film thickness and coating materials has been developed and the coatings can be classified into two groups: firstly the pure liquid polymer coatings including polydimethylsiloxane (PDMS) and polyacrylate (PA); and secondly, mixed films containing liquid polymer and solid particles such as Carboxen-PDMS, Divinylbenzene (DVB)-PDMS, Carbowax-DVB and DVB-Carboxen-PDMS (Pillonel, Bosset, & Tabacchi, 2002).

The main advantages of SPME are in reducing the amount of solvent used as well as the combining of sampling and extraction into one step. It may be used for polar and non-polar analytes in a wide range of matrices and high sensitivity can be achieved. Some disadvantages have been encountered including batch to batch variation and robustness of fibre coatings (Ridgway, Lalljie, & Smith, 2007).

3.10 Chromatographic separation

Currently, acrylamide is most commonly analysed by GC or LC and for detection, GC can be coupled with flame ionization (FID), ECD or MS whereas LC involves MS. Most commonly, GC has been used in the quantification of acrylamide in a variety of industrial and environmental applications with the main advantages being relatively low cost and efficient detection of semi-volatile compounds.

It has been reported that care should be exercised in the choice of column. Because acrylamide is a strongly polar molecule, non-polar columns including DB-5, DB-23, HP-5 are not suitable for analysis (Zhang et al., 2006). By contrast, Gökmen et al. (2005) demonstrated that the retention of acrylamide on columns could be improved by avoiding organic components such as acetronitrile and methanol in the aqueous mobile phase.

3.10.1 Gas chromatography using electron capture detection (GC-ECD)

The use of GC-ECD was first applied to analysis of acrylamide contents by Zhang et al. (2006). It was shown that no further clean up was required after derivatisation and sensitivity was slightly higher than obtained with MS/MS-based methods. GC-ECD has been described as one of the most sensitive GC systems available and this shows an enhanced and selective response to a halogen group, a nitro moiety and carbonyl-containing compounds (Flanagan, Taylor, & Watson, 2008). The ECD is a selective detector that provides very high sensitivity for the compounds that “capture electrons”. This detector has been used widely for the analysis of pesticide residues, as well as samples of wildlife whole blood and wildlife feedstuffs (McNair & Miller, 2009).

GC-ECD has been applied for the determination of acrylamide in fried foods by Zhang et al. (2006). This involved a polar column and the results showed accuracy, reliability and suitable for routine acrylamide analysis. In addition, GC-ECD was used for the qualification of acrylamide in heated-processed starchy foods. GC coupled with ECD was performed on a polar column and achieved a good separation, convenience and reliability for food products (Zhu et al., 2008).

3.10.2 Gas chromatography-mass spectrometry (GC-MS)

Acrylamide has been examined by GC-MS in various foods with or without the use of derivatisation. There are a limited number of articles concerned with the technical aspects of the measurements: although GC-MS are widely used for acrylamide determination, it has been suggested that method validation, extraction and clean-up procedures still need to be optimised (Zhang, Zhang, & Zhang, 2005). From the current literature, it appears that GC-MS after bromination is an effective choice of analysis with a detection level lower than 10 μg/kg. MS offers the advantage of a high degree of specificity as it is concerned with vapour-phase separation of ionized fragments according to their mass-to-charge ratio (*m/z*) (Flanagan et al., 2008).

GC-MS has been utilised to detect acrylamide in cooked rice, tomato sauces and some fast foods on the Italian market without derivatisation in single ion monitoring mode. The GC column was a Supelcowax^{TM}-10 fused silica capillary column (30m × 0.25mm i.d., 0.25 μm film thickness). The limit of detection (LOD) was found to be 25 μg/kg and limit of quantitation (LOQ) was 75 μg/kg and satisfactory results were obtained (Tateo, Bononi, & Andreoli, 2007). In addition, French fried and baked corn chips were examined with GC-MS with derivatization and the column used was a nonpolar column (PE-5, 30m × 0.25mm, 0.25 μm i.d.). Identification of brominated acrylamide involved monitoring of peaks at m/z $[C_3H_5{}^{81}BrNO]^+$ = 152, $[C_3H_5{}^{79}BrNO]^+$ = 150, $[C_2H_3{}^{79}Br]^+$ = 106 and $[C_2H_3{}^{81}Br]^+$ = 108 (Jung, Choi, & Ju, 2003).

In summary, there have been a number of published reports on the analysis of acrylamide in various foods. Various approaches have been used and suitable approaches described for particular foods that contain acrylamide. To date, no single procedure for extraction, preparation as well as analysis has been found to apply to all foods. In addition, no single approach has gained the status of a standard method and it has previously been suggested that validation of methods for acrylamide analysis is an important priority (Zhang et al., 2005).

3.11 Introduction to strategies to reduce acrylamide content in processed foods

The available information on acrylamide raises questions regarding its significance in food products and whether current intakes are sufficiently high to place consumers at

risk. Whilst it is difficult to answer these questions, a consensus approach has been developed and this has involved consultation between organisations with a range of different interests in food safety. A relatively conservative approach is being applied so that processors are taking steps to minimise acrylamide formation in order that intakes will be reduced as far as possible. The position applying in Australia and New Zealand is similar to that adopted in Europe and North America has been described by the Food Standards organization (Food Standards ANZ, 2010a). This promotes use of toolbox resources such as that produced by the Confederation of the Food and Drink Industries of the EU (known as the CIAA) (CIAA, 2010)

In the context of the need for strategies which might be used in commercial processing of foods, a particular focus of research has been the impact of processing conditions upon formation of acrylamide especially in foods made from potatoes.

The first consideration has been to examine the factors that are already known to affect the Maillard reaction and from this a series of strategies to reduce for minimisation of this reaction have been formulated. The factors include conditions of temperature and time during processing, composition of the food ingredients, moisture content, pH as well as concentrations of reactants. This has allowed the identification of a variety of approaches to be used to reduce acrylamide levels, including removal of the reactants (asparagine and reducing sugars) as well as reducing processing temperature and time.

3.12 Impact of raw materials

Acrylamide is primarily generated by the reaction between the primary precursors asparagine and reducing sugars, during the Maillard reaction. In potatoes, reducing sugars can be considered to limit acrylamide formation whereas asparagine is more abundant (Claus, Mongili, Weisz, Schieber, & Carle, 2008). It has been found that the asparagine contents of raw potatoes vary only within a narrow range whereas reducing sugars are much more varied as these depend on variety, storage condition as well as other factors (Amrein et al., 2004b). For cereal based foods, the asparagine level in the raw material together with the method of processing appear to be the primary determinants of the amount of acrylamide in final products (Codex Alimentarius Commission, 2007). In a study of French fries, Brunton et al. (2007) found linear

relationships between reducing sugars (including both fructose and glucose) and acrylamide content with R^2 values of 0.65 and 0.68, respectively. However, no significant correlation was observed between the contents of asparagine and acrylamide. In addition, a significant correlation existed between acrylamide content and colour characteristics (*L* and *b* values) measured using a Hunter meter and as the colour of French fries became darker, the acrylamide content increased.

Based upon compositional studies of bread wheat (*Triticum aestivum*) it might be predicted that there is a likelihood of acrylamide formation, which is higher than that of other cereal grains including maize and rice although it is less than for potato products (Curtis et al., 2009). In bakery foods, flour is the main source of asparagine and the amounts of the free amino acid have been measured (Table 3.2). In a study of 75 different flours the free amino acids found in virtually all samples were aspartic acid, asparagine, glutamic acid, tryptophan, alanine and glutamine (Amrein, Andres, Escher, & Amadò, 2007).

At least some quality attributes of wheat flours also appear to influence the extent of formation of acrylamide (Claus et al. 2006). Flour extraction rate during milling, as indicated by the ash content, appeared to affect acrylamide contents in bakery products. Acrylamide content was almost double in wafers made using type 1050 flour (1.05% ash) compared to 550 flour (0.55% ash). This may reflect the higher ash content or higher concentrations of asparagine in the outer layers of the grain. Claus et al. (2006) also evaluated the influence of environmental conditions as well as genetic varieties on acrylamide contents. Growing conditions, use of fertilisers as well as genotype of the wheat were all found to impact on acrylamide content of the resultant baked products.

3.13 Impact of formulation and additives

Although modifications to formulation may result in sensory changes to final products thereby influencing consumer acceptance, this approach is a relatively simple and efficient method for minimisation of acrylamide in foods (Claus et al., 2008).

One strategy has been the addition of free amino acids other than asparagine: lysine, glycine and cysteine have been trialled and these were found to decrease acrylamide in wheat-flour products. In commercial situations, addition of amino acids, citric acid, antioxidants and emulsifiers can effectively reduce acrylamide content. Glycine, L-lysine and L-cysteine decreased the formation of acrylamide by 95%, 91% and 87%, respectively while L-glutamic acid had a lower effect, giving a reduction of less than 20% (Kim, Hwang, & Lee, 2005).

In sweet bakery products, raising (aerating) agents, reducing sugars and organic acids are the components that appear most likely to be useful for manipulation of acrylamide formation. For example, in biscuits, the replacement of ammonium hydrogencarbonate by sodium hydrogencarbonate reduced the acrylamide content by approximately 70% and using sucrose instead of invert sugar syrup gave a similar effect. Similarly, ammonium hydrogencarbonate gave the highest content of acrylamide in gingerbread products (Graf et al., 2006).

Acrylamide content has been found to be dramatically reduced by addition of citric acid prior to frying of French fries. The application of 0.2% citric acid, induced 82.2% and 72.8% reduction of acrylamide formation in fried and baked corn chips, respectively (Jung et al., 2003).

3.14 Impact of frying time and temperature

Deep frying is defined as the immersion of a food product in edible oil heated above the boiling point of water (Hubbard & Farkas, 1999). Optimisation of the frying process has the potential to control the kinetics of both the browning reaction as well as acrylamide formation. Amrein, Limacher, Conde-Petit, Amadò, and Escher (2006) found longer

heating times at all temperatures and higher moisture contents in a potato model system resulted in increases in browning and also the formation of acrylamide.

In potato products, a reduction in the surface area corresponded to slower heating of the strips during frying. For the same frying time, the acrylamide level in larger strips is lower but a longer time of frying is required for the interior of the strip to be cooked. Gökmen and Palazoğlu (2008) found that the acrylamide level for the same frying temperature (170°C), time (5 min) and reducing sugar content (1.5 g/100g dry matter) of a 10 mm strip (980 µg/kg) was lower than a 8.5 mm strip (1502 µg/kg).

Due to its small size (71 Da), the molecules of acrylamide can be removed from the foods as vapour and selection of suitable temperature and pressure conditions can be used to facilitate such losses (Anese, Suman, & Nicoli, 2010). Acrylamide contents have also been found to decrease with increasing water activity and thus water appears to play an important role in influencing acrylamide removal. For a constant frying time, the acrylamide content increased with increasing temperature following an exponential function while at a constant frying temperature, acrylamide increased linearity with a greater slope at higher temperature (Gökmen, Palazoğlu, & Senyuva, 2006). Williams (2005) found a highly significant relationship between acrylamide content and both the time and temperature of cooking. Soaking in water before frying and the type of oil were found to be not significant. Although changes in the peroxide value were observed during frying, no significant relationship to acrylamide formation was established (Williams, 2005).

3.15 Impact of water activity

Well before the discovery of acrylamide in foods, water activity had been shown to be a crucial factor in controlling the Maillard reaction (Ames, 1990; Labuza & Saltmarch, 1981). At low a_w, the mobility of reactants is limited, despite their presence at increased concentrations. The Maillard reaction occurs most rapidly at intermediate a_w values (0.5-0.8) although the specific relationships between reaction rate and a_w depends upon the particular food system.

In relation to browning, rates are affected by other components including glycerol and these can lower the a_w at which maximum browning occurs (Ames, 1990). According to Eichner and Karel (1972), the browning rate decreased with increasing water content, although the mobility of reactants became substantially impeded at low water contents. The role of water on the sugar-amino browning reaction in a food system is complex. At higher a_w, the reaction rate decreases due to dilution of the reactants while at low a_w, the amount of mobile water is reduced and as a result there is a decrease in the reaction rate. Similarly, Mestdagh, De Meulenaer, Cucu, and Van Peteghem (2006) found that at moisture values above 100% (expressed on a dry weight basis) acrylamide contents decreased significantly in a potato model system. In addition it was observed that acrylamide content was more dependent upon the moisture content than on a_w in the high-moisture potato powder model system. Typically, the rate of browning reactions in foods increases with increasing a_w up to a maximum and then falls at even higher a_w values (Labuza & Saltmarch, 1981).

3.16 Impact of pH value

One of the important factors reported to determine the rate and extent of formation of Maillard reaction is the pH conditions in a food (Nursten, 2005). Specific evidence is now accumulating that acrylamide formation is also pH dependent and lowering the pH of food is inhibitory (Pedreschi, Kaack, Granby, & Troncoso, 2007). In a study of fried corn chips, Jung et al. (2003) found that reducing pH by adding a small quantity of citric acid before frying can decrease acrylamide formation. Adding citric acid at levels of 0.1% and 0.2% significantly inhibited acrylamide formation by 58 and 73%, respectively.

Huang, Yu, Zou, and Tilley (2008) investigated the effect of frying time and temperature as well as pH for products prepared by deep frying of a wheat flour dough. The acrylamide content was found to increase as dough pH increased, reaching a maximum at pH 7.2. This can be due to the blocking of the reaction between asparagine and the carbonyl group at low pH. Frying temperature and time also had a significant effect on acrylamide.

3.17 An overview of asparaginase as a strategy for mitigation

Among the various strategies that have been considered for minimising the overall intakes of acrylamide by consumers have been ways of reducing the amounts of precursor compounds. There is genetic variability in at least some species including potatoes which might be exploited by plant breeders (Kornburst et al., 2009). Another quite recent proposal for removing free asparagine in food formulations is an enzymatic approach. This utilises asparaginase which hydrolyses asparagine to products which do not contribute to acrylamide formation (Kukurová, Morales, Bednáriková, & Ciesarová, 2009).

The asparaginase enzyme is now available commercially and production is based on cloning of fungal enzymes from *Aspergillus* sp. The enzyme is isolated from a fermentation broth by filtration to remove the biomass and concentrated by ultrafiltration and/or filtration. As yet, only limited information is available on the applications of the commercial enzyme although the manufacturers have now recommend dosages reflecting the products, type of flour, formulation, process and the extent of reduction in acrylamide being sought (Novozymes, 2010) and a copy of the specification sheet provided by Novozymes is presented as Appendix 4.

3.18 The principle of asparaginase

Asparaginase [L-asparagine amidohydrolase, EC number 3.5.1.1 (Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, 2011)] catalyses the hydrolysis of the amide group of the side chain of asparagine yielding an ammonia molecule and one of aspartic acid (Figure 3.5). The latter can then be readily converted to oxaloacetate which enters the citric acid cycle so that the primary precursor of acrylamide (asparagine) is eliminated from the food system before heating commences.

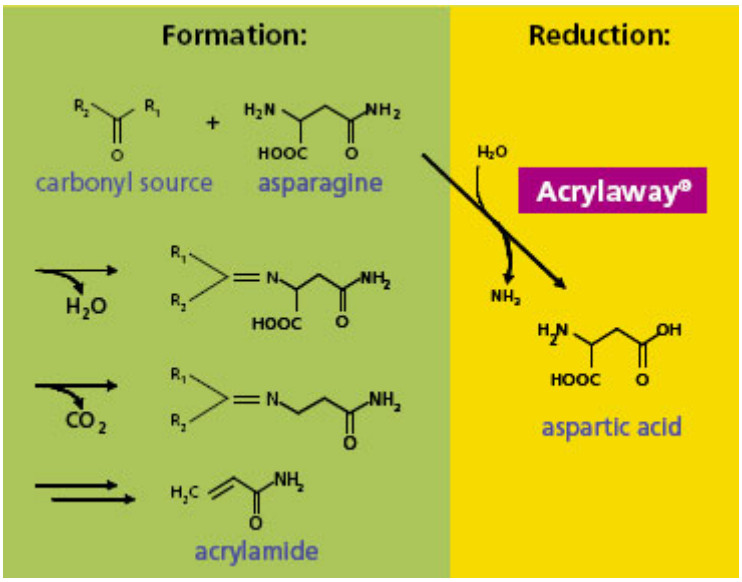


Figure 3.5 The reactions involved in removal of precursor asparagine by the enzyme asparaginase

Notes 1 Acrylaway® is the trade name for asparaginase used by Novozymes
2 Source of information was Novozymes, 2010

Commercial asparaginase preparations have been developed and two are now available for the reduction of acrylamide. These are PreventASe ^{TM72} from DSM and Acrylaway® from Novozymes. Both enzymes have been cloned from fungal species which have been classed as generally recognised (or regarded) as safe (GRAS). Acrylaway® was produced by analysing gene sequence from *Aspergillus oryzae* whereas PreventASe was developed from *A niger* (Kornburst et al., 2009). These commercial enzymes have been characterised and that from *A oryzae* has a pH optimum at 6-7 with good activity between pH 5 and 8 (Pedreschi, Kaack, & Granby, 2008) while at pH 7 the optimum temperature is 60°C. The preparation from *A niger* is reported to have a more acidic profile. Acrylaway® is sold in the forms of both liquid and granulate which have the same strength (3500 ASNU/g) and the molecular weight of the enzyme was determined to be 36 kDa (Food Standards ANZ, 2010c).

3.19 Application of asparaginase in food products

The asparaginase preparations have only become commercially available quite recently and therefore there have been very few studies published on these in the scientific literature. In an early report that used an enzyme from a laboratory source, Ciesarova,

Kiss, and Bogl (2006) found that incubation of an L-asparaginase with fresh potato mash at a temperature of 20°C resulted in a reduction of around 50% in the acrylamide content after frying.

It has been reported that treatment is not a simple undertaking because each product contains a variety of food components and these may influence enzyme activity and reactivity. In addition, the rate of enzymatic hydrolysis of asparagine is dependent upon physical processing parameters including temperature, pH, water activity and time. Furthermore, the content of precursors varies in the food products and the reaction-limiting factor differs from product to product (Kornburst et al., 2009).

It is claimed by the manufacturer that Acrylaway® can be successfully used to decrease the acrylamide content in a wide range of cereal-based foods including biscuits, pretzel, crisp bread and ginger biscuits. Reductions up to 90% may be achieved without changing the taste and appearance of a final product. In semisweet biscuits treated with asparaginase, a significant reduction of acrylamide in the final biscuits was achieved compared to the control (Novozymes, 2010). In one of the few published reports, Pedreschi et al. (2008) showed a 30% reduction in acrylamide formation for potato strips after treatment with Acrylaway® at 40°C for 20 min.

3.20 Summary of current knowledge on acrylamide in foods

This chapter has presented a brief overview of acrylamide in terms of the mechanism of formation, analytical techniques as well as some possible approaches that might affect acrylamide formation in various foods especially cereal-based products. The current literature on acrylamide confirms that a number of challenges confront the analyst seeking to measure acrylamide as well as processors who attempt to minimise formation of the toxin.

In the relatively short time since 2002 when acrylamide in foods became an issue of concern, most of the published research has focussed upon potato products. As lower concentrations of acrylamide have been found in cereal-based foods compared to potato products, the optimisation of analytical techniques for products made form grains warrants further attention particularly to ensure a suitable level of sensitivity.

Although acrylamide formation might be expected during manufacture of Asian instant noodles, there is only very limited data available on this. In the context of pressures on manufacturers to minimise the potential risks that might be associated with even moderate intakes of acrylamide, further emphasis upon process conditions is also required. This can include optimisation of frying time and temperature as well as water content and pH conditions. In addition, the application of asparaginase is a new alternative for reducing acrylamide contents and this warrants further investigation. The potential application of this approach for instant noodles has not yet been reported.

Chapter 4

Background and literature review: The processing of instant noodles and their global significance as a food source

The purpose of this chapter is to provide background and review current knowledge on the utilisation of wheat flour particularly regarding the processing of instant noodles. The areas covered include a description of the raw materials used in manufacture of these products.

4.1 Introduction to instant noodles

Asian noodles and Italian pasta differ in raw materials and processing procedures. Noodles are usually made from wheat flour by a process of sheeting and cutting as opposed to pasta products which are processed from coarse semolina from durum wheat by extrusion (Fu, 2008). Instant noodles are the fastest growing segment of the noodle industry and play an important role as a popular and increasingly widely consumed product in many Asian countries (Hatcher, 2001). The term instant noodles refers to one of the more recent developments in the long history of Asian noodles. Instant noodles are usually steamed and deep-fat fried products (Kim, 1996) and are called ramyon in Korea and ramen in Japan. These were first produced commercially in Japan during 1958 and in Korea in 1963 (Kim, 1996). Recently, instant noodles are being consumed in more than 80 countries and have become an international food. Their popularity reflects the ease of preparation, only requiring brief cooking or steeping in boiling water, as well as their advantages of light weight, long shelf life, low cost and the availability of a wide range of flavours. Instant noodles have distinctive flavour and colour characteristics because of the unique processing steps applied in manufacture, particularly those of steaming and frying (Park, Shelton, Peterson, Kachman, & Wehling, 1997).

4.2 Raw material of instant noodles

The primary ingredients of instant noodles are wheat flour, salt (1.5-2.0% based on wheat flour weight) and water (Kim, 1996). The other ingredients commonly used

include the alkaline salts, typically an equal mixture of sodium carbonate and potassium carbonate and the concentration of alkaline salts is around one-tenth of the amount of salt although high-quality forms of the product may be produced without this ingredient (Kim, 1996). Phosphates, guar gum, riboflavin, sorbitol and oil such as canola oil, are also used (Kim, 1996). Guar gum is added to noodle formulations to enhance the elasticity and extensibility of cooked instant fried noodles (Yu & Ngadi, 2004). Oil and sorbitol are reported to have a role in emulsification and riboflavin is used to enhance the colour and appearance of the product (Kim, 1996).

4.2.1 Wheat flour

Wheat is a member of the grass family (*Gramineae*) that is grown in spring and winter and is the most cultivated cereal grain in more than 120 countries around the world (Collado & Corke, 2004). Wheat is regarded as the most important cereal in the world in terms of production and value for human food and animal feed. It makes the largest contribution to food energy in human diet and is used worldwide as a human food, an animal feed and an industrial feedstock. It is primarily used in bread products, pasta, noodles, pastry, breakfast cereals and baby food. Wheat is regarded as having many advantages including its nutritional value and the ability to be stored, transported and processed to produce highly refined foods (Hui, 2006). All wheats belong to the genus *Triticum* of the family *Gramineae* and the main species grown around the world are *Triticum aestivum* and *durum* (Hui, 2006). Durum is noted for its extreme hardness and is generally used for making pasta and couscous. The Australian Bureau of Statistics (ABS) reports that durum production typically comprises less than 3% of the total Australian wheat production (ABS, 2008).

In contrast, wheat flour (from *T. aestivum*) is one of the main ingredients of noodles and the quality characteristics of flour influence the attributes of the final products. The key is to choose wheat with the most suitable qualities in order to make an appealing finished product. There are many flour types that may be used in instant noodle production and flour quality affects the colour, opaqueness, strength and cooking quality of noodles (Oh, Seib, & Chung, 1985b).

For the purposes of storage and marketing Australian wheat is classified into six major categories and the classification is based upon variety, degree of soundness (that is, lack of sprouted grain), cleanliness (the lack of foreign materials, other type of grains and weed seeds) and protein content (Bach, 2009; Martin & Stewart, 1994). The classification is shown in Table 4.1.

Table 4.1 Major Australian wheat grades and their typical end-uses

Grade	Production (%)	Typical protein content (%)	End uses
Australian Prime Hard	<5	13-14	Used for Japanese style ramen noodles and Chinese-style yellow alkaline noodles
Australian Hard	15-20	11.50	Suitable for a wide range of breads and Chinese-style alkaline noodles
Australian Premium White	30-35	10	To produce a variety of Asian noodles including Hokkein, instant and fresh noodles
Australian Standard White	20-30	9-10	Suitable for a wide range of products including Middle Eastern, Indian and Iranian-style flat breads and Chinese steamed bread
Australian Noodle Wheat		10.50	Specially developed for use in noodle industry especially in Japan and Korea
Australian durum Wheat	<5	10-13	Suited to the production of pasta

Note Sources of data used were AWB, 2005; Bach, 2009; Martin & Stewart, 1994

4.2.2 Water

Water plays an important role in noodle making and it has a significant effect on the quality of final products. Water is added to the wheat flour with kneading to form dough as a result of the formation of a gluten network which contributes to the noodle structure. Water also contributes the viscoelastic properties of the dough and increases

the smoothness of the texture of the final cooked product. The amount of water needs to be optimised so there is sufficient water to hydrate the flour and allow development of a uniform dough sheet (Fu, 2008). If higher amounts of water are added, a very soft dough is formed. If an amount of water less than 35% is added, the dough becomes resistant and as a result a longer time is taken to form the dough.

4.2.3 Salt and alkaline salts

Salts (including sodium chloride as well as alkaline salts) have a significant role in noodle making, acting to toughen the dough during mixing and sheeting and to modify processing, dough and starch properties. The commonly used term kansui refers to alkaline salts that are potassium carbonate, sodium carbonate, phosphates of sodium and potassium as well as combinations of these. Other alkaline reagents including sodium hydroxide and bicarbonates are also used in some countries depending upon local preference. Kansui interacts with the gluten and produces a gumlike texture, yellow colour and enhances the flavour. In addition, the alkaline salts increase the pH thereby extending the shelf life, while inhibiting enzyme activities and the growth of micro-organism. The change in pH conditions probably decreases the activity of the deteriorative enzyme polyphenol oxidase thereby improving colour stability (Miskelly, 1998). Moreover, it also enhances flavours and texture while reducing the boiling time of instant noodles (Kubomura, 1998).

4.3 Ingredient formulations for instant noodles

Based on the earlier review by Hou and Kruk (1998), the ingredients used to make instant noodles are flour, water, salt, potato starch, sodium carbonate, potassium carbonate, guar gum and polyphosphates. The typical ingredient formulation used in the commercial production of instant noodles is presented in Table 4.2.

Table 4.2 Formulation for instant noodles

Ingredients	Amount (%)
Wheat flour	100
Distilled water	34-37
Salt (NaCl)	1.6
Alkaline salts (potassium : sodium carbonate 6:4)	0.2
Potato starch	0-12
Guar gum	0-0.2
Polyphosphates	0-0.1

Notes 1 Values are expressed with respect to the amount of flour
2 Source of data used was Hou and Kruk (1998)

4.4 The processing of instant noodles

The processing of instant noodles does differ from country to country and also from individual company to company but the basic principles are the same (Kim, 1996) and the series of steps typically applied in commercial processing are shown in Figure 4.1.

4.4.1 Mixing steps

The ingredients other than the flour are predissolved in soft water and stored in a storage tank. This mixing water has a pH around 7.2-7.5 and temperature around 20-30°C. Wheat flour is mixed with the water in which the salt and alkaline salts have been predissolved. In order to achieve high-quality, mixing time is 5-7 min at high speed and 18-20 min at low speed is used. After water is added to the flour and mixing proceeds, the dough becomes very soft and sticky and the gluten structure is gradually developed and shows elasticity. The ingredients are uniformly mixed in this step and the flour particles become hydrated (Kim, 1996). As part of this process, flour protein molecules bind water strongly and as a result small dough crumbs are formed (Azudin, 1998).

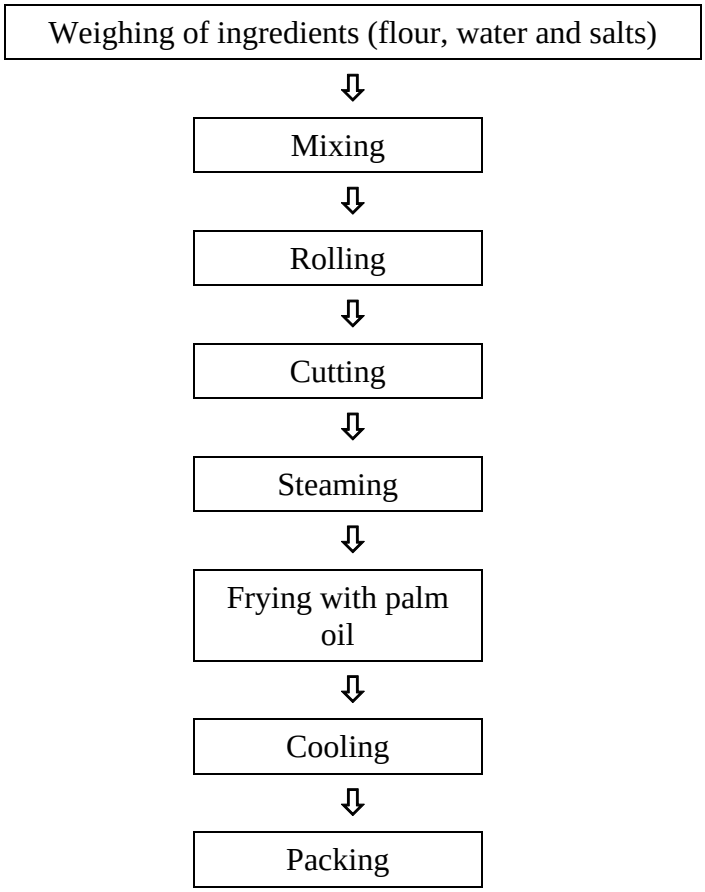


Figure 4.1 Process diagram manufacture of instant noodle

Note Source of information was Kim, 1996

The crumb size is important in the production of noodle dough sheets because large crumb sizes lead to uneven dough sheets and are associated with uneven hydration. As a result, dough sheets may be subject to tearing during the sheeting stages (Azudin, 1998). High protein flour tends to create larger crumbs and in commercial situations, the crumb diameters are approximately 3 cm (Azudin, 1998).

The mixing step is often followed by a period during which the dough is rested. This step allows time for further hydration of flour particles and allowing water to redistribute in the dough system. Moreover, the resting step can also have the effect of enhancing gluten formation during the subsequent sheeting steps (Fu, 2008).

4.4.2 Rolling and noodle formation

The crumby dough pieces are compressed by pairs of rolls and folded (Kim, 1996). Although the development of the gluten matrix occurs during the mixing, it is still far from complete at the end of the mixing stage. The continuous protein matrix is formed in the rolling stage (Azudin, 1998). The gap between the two smooth metal rolls is used to control the thickness of the sheet and the difference in speed between the rolls is responsible for feeding the noodles through the plant and results in the formation unique wave pattern of characteristic of instant noodle strands (Kim, 1996). During rolling the gluten matrix becomes aligned along the direction of sheeting. The using of multi-roller sheeting with waved rollers has been a significant breakthrough and the network structure of gluten increases the quality of noodle products (Wu, Kuo, & Cheng, 1998). The roll speed decreases as the surface firmness increases and the reduction of constant roll speed leads to increasing firmness (Oh, Seib, Deyoe, & Ward, 1985a).

Once the dough sheet is reduced to the desired thickness, it is cut into noodle strands along the direction of sheeting. The shape and width of noodle strands are determined by the dimensions of the cutting rolls (Fu, 2008).

4.4.3 Steaming

The resultant wavy noodle strands are steamed in a steam chamber and this effectively results in the cooking of the noodle (Kim, 1996). The starch gelatinises and this, coupled with the swelling of the starch gel system to the full extent, ultimately bestows a pleasing texture (Wu et al., 1998). During these changes, the gluten is thought to remain as a fixed permanent structure and during steaming starch gelatinisation probably begins as the temperature rises above 80°C. However, it has been reported that excessive swelling of starch on the noodle surface can cause many processing problems (Wu et al., 1998).

4.4.4 Frying

Generally the dimensions of a fryer in a commercial plant are approximately 10 m long and 1 m wide and the noodles are dehydrated during this step (Kim, 1996). Palm oil, beef tallow or a combination of these are commonly used as the frying medium in most

South East Asian countries although in Korea palm oil is used extensively (Hui, 2006) and instant noodles have a shelf life of 4-6 months (Miskelly, 1993). The temperature is constantly maintained by a heat exchanger and the frying conditions are 60-70 second at 145-150°C and 90-120 s at 157-160°C, respectively for bag and cup noodles (Kim, 1996). The purposes of frying are to remove excess water, to incorporate oil into the noodles, to allow starch to become gelatinised and create the desired structure, both externally as well as internally where a porous structure is formed (Kim, 1996). This is created through due to the steam vapourisation and subsequent transfer of oil into the open pores on the noodle surface from which the steam escaped. The degree of starch swelling and moisture gradient in the noodles play a crucial role in determining the noodle texture. Complete swelling of starch is associated with fried instant noodles having a good texture after being cooked in boiling water (Wu et al., 1998). Water also plays an important role in the transfer of heat into the noodles. During frying, water in noodles migrates from the central portion outwards to replace that lost by dehydration on the exterior surface.

4.4.5 Packaging

The fried noodles require immediate cooling to room temperature in a travelling cooling tunnel to avoid rapid oil oxidation and to facilitate the drainage of excess oil away from the surface of the product (Kim, 1996). Then, the noodles are packed into bags using an automated system and the packaging materials commonly applied are polypylene/polyethylene film for the common noodle and polypropylene/polyester/polyethylene for high-quality noodles (Kim, 1996).

4.5 Current knowledge on niacin and acrylamide content of instant noodles

Currently, there is relatively little data available on the nutritional value of instant noodles. Some recent research has specifically focused on other vitamin groups including thiamin, riboflavin, folic acid as well as ascorbic acid in Asian noodles products (Bui & Small, 2007a, 2007b, 2007c, 2007e, 2008a, 2008b, 2009; Hau Fung Cheung, 2008; Hau Fung Cheung et al., 2007, 2008, 2009; Ma, Cato, & Small, 2007; Sanyoto, Wijaya, & Small, 2008). These studies have been reviewed briefly in Section 2.1 and have shown that there are considerable losses of these vitamins during processing, preparation as well as storage in the case of fresh noodles. Some of these

studies have also demonstrated that losses impact both on the naturally occurring forms of the vitamins as well as those forms added as fortificants.

To date there have been no comparable studies of niacin or niacin fortification of Asian noodles, including instant forms. However, there have been some studies on niacin content of other types of food including infant formulas, soybean, rice, dried bread, barley, coffee, soft drink and pork products (Juraja et al., 2003; Perrone, Donangelo, & Farah, 2008; Saccani, Tanzi, Mallozzi, & Cavalli, 2005; Schreiner, Razzazi, & Luf, 2003; Woollard, 2002). These have typically been focussed upon the challenges of analysis in complex matrices. Therefore it is still not clear whether there might be significant losses in niacin during processing and storage of instant noodles. The consumption of instant noodles has been increasing particularly in the last decade so that globally there is a high demand for the products. On this basis, these might be considered as potential vehicles for fortification and enhancement of dietary intakes of essential nutrients. Therefore the addition of niacin to noodles warrants study so that the stability and retention can be evaluated.

On the other hand, acrylamide has been classified as probably carcinogenic to humans and since its discovery in foods there has been considerable research undertaken on a variety of foods. This has emphasised procedures for analysis, the chemical mechanisms whereby acrylamide forms as well as strategies for minimisation of its formation. Much of this work has employed either model systems or has been directed towards the production of fried foods especially those made from potatoes. Currently, there is very little data available on analytical procedures validated for instant noodle samples and the levels likely to form in these products remains to be elucidated.

Chapter 5

Summary of background and description of the project aims

The purpose of this chapter is to summarise the context in which the current project has been developed and to describe the aims.

5.1 Summary of current situation and significant of the project

The production and consumption of instant noodles has increased dramatically in the last decade because of convenience, light weight, long shelf life, low cost and the wide range of flavours available. In addition, instant noodles can be considered as a staple food in a number of Asian countries and consumption continues to expand in many countries around the world, including Australia. With this growing popularity, instant noodles might be considered as a vehicle for vitamin fortification, although further studies are needed if we are to fully understand the retention of vitamins in these foods, particularly of niacin. A further consideration is that instant noodles may contribute significantly to the intake of acrylamide by at least some consumers as the products are carbohydrate-rich and frying occurs at quite high temperatures during manufacture. There have been virtually no previous research into these components, their contents and factors influencing these in instant noodles, therefore this project was developed in order to extend our knowledge in theses areas.

5.2 Hypothesis

This project has been based upon the hypotheses that:

1. Instant noodles might be a suitable vehicle for fortification with niacin so that these products can provide a supply of this nutrient in the diet; and

2. Optimisation of processing conditions as well as the incorporation of the enzyme asparaginase might be used to control acrylamide formation in the processing of instant noodles.

5.3 Project aims

The broad aims of the project have been to investigate the analysis of instant Asian noodles and the influence of processing as these relate to niacin and acrylamide. The specific objectives have been to:

1. Evaluate procedures for the extraction and analysis of niacin for samples of instant noodles, so that a reliable procedure validated and suitable for instant noodles is available;

2. Investigate the impact of processing and process parameters on niacin contents during preparation of instant noodles;

3. Determine the potential of instant noodles to provide a vehicle for fortification in order to enhance niacin intakes and ensure their adequacy;

4. Assess and compare suitable methods for both extraction and measurement of acrylamide in instant noodles and to adapt and validate these to ensure reliability and repeatability for their application in this research;

5. Analyse the acrylamide contents in a range of commercial instant noodle products in order to assess the range of amounts currently present in these foods;

6. Monitor and investigate the factors impacting on the acrylamide content of instant noodles, to include time and temperature of frying as well as pH and other relevant variables; and

7. Investigate the potential application of the enzyme asparaginase and interactive effects of asparaginase and processing parameters, as a basis for recommending strategies for reducing acrylamide formation in Asian instant noodles.

Chapter 6

Materials and methods

The purpose of this chapter is to introduce the chemicals, reagents, instrumentation and methods used during this study. This includes procedures applied in the sampling and preparation of noodles, as well as methods used in the analysis and further investigations into niacin and acrylamide in instant noodles.

6.1 Materials

All chemicals used in product formulations and analytical procedures were of analytical grade or of the highest purity available unless otherwise specified. Nicotinic acid and nicotinamide were used in the current study as chemical standards for analytical purposes and a reference sample was used for method validation and in the assessment of extraction procedures (Table 6.1). The specifications supplied with the reference sample are shown in Table 6.2. In addition the details of the reference sample used for validation of the extraction and analysis of acrylamide are presented in Tables 6.3 and 6.4. Details for other chemicals used in the niacin and acrylamide studies with the respective suppliers are listed in Tables 6.5 and 6.6, respectively.

In this study, water was used in a procedure including the preparation of solution of standards, reagents and all HPLC and GC solvents. Water also used in the preparation of extracts acrylamide content in instant noodles. In all cases the water used was of milliQ grade and the term water is used in this thesis to specify milliQ water.

The details of commercial flours used for making instant noodles are listed in Table 6.7. Seven commercial instant noodles were purchased from the shops in Melbourne and these were selected so that different origin countries were represented (Table 6.8).

6.2 Apparatus and auxiliary equipment

The items of equipment and analytical instruments used along with the details of manufacturers as well as model numbers are listed in Table 6.9. The HPLC and GC

system components and ancillary items used are presented in Tables 6.10, 6.11, 6.12 and 6.13, respectively.

Table 6.1 Details of vitamin compounds and the vitamin reference sample

Sample	Description	Supplier
Nicotinic acid	72309, 32107022	Fluka Biochemika, Germany
Nicotinamide	N-3376, 120H0161	Sigma, USA
AACC reference sample	Ground-up fortified ready-to-eat cereal, Sample number VMA 406	AACC, St Paul, MN, USA

Notes 1 Description presented as product number, batch or lot number
2 AACC refers to the American Association of Cereal Chemists International

Table 6.2 Specifications for niacin in the AACC reference sample

Vitamin	Mean (mg/100g)	Number of analyses	Standard deviation (sd)	Coefficient of variability (%)	Range of vitamin contents (mg/100g)
Niacin	26.51	8	3.73	14.1	20.93-32.85

Notes 1 The values in this table are taken from those provided with the sample and represent the results obtained by the laboratories which originally participated in the check sample collaborative survey run by AACC.
2 A copy of the complete certificate provided by AACC is presented as Appendix 1

Table 6.3 Specifications for niacin in the Kellogg's Special K

Vitamin	Quantity per serve	Quantity per 100g
Niacin	25 mg (25%)	8.3 mg (83%)

Notes 1 The values in this table are those provided on the package of the product purchased commercially and used in the validation of the niacin analysis procedures
2 The values in parentheses are the proportion of the Australian Recommended Dietary Intake value represented by the niacin present (Food Standards ANZ, 2010b)

Table 6.4 Specification for the acrylamide content in the NIST sample

	Mass fraction (ng/g)
Acrylamide	87.0 ± 7.8

Notes
1 Product code is SRM 2387 - Peanut Butter
2 NIST refers to the National Institute of Standards and Technology
3 The values in this table are those provided with the sample and represent the results obtained by the laboratories which originally participated in the check sample collaborative survey run by NIST
4 A copy of the complete certificate provided by NIST is presented as Appendix 2

Table 6.5 Details of chemicals used in the study of niacin

Chemicals	Supplier
Barium hydroxide (D3247, 002238) Calcium hydroxide(124) di-sodium hydrogen orthophosphate (dehydrate) (D3247, 61280) Sodium hydroxide pellets (1310-73-2, AF702293)	Ajax Chemicals International Pty. Ltd., Level 24, 270 Pitt St, Sydney, NSW, Australia
Methanol (67-56-1, FCFM1H)	Burdick and Jackson, USA
Oxalic acid (10174, 58809) Sodium acetate (anhydrous) (10236, 20194)	BDH Chemicals, Australia Pty. Ltd., 207 Colchester Rd, Kilsyth, VIC, Australia
Potassium di hydrogen ortho phosphate (392)	By-products & chemicals Pty,Ltd, Sydney, Australia
Acetic acid Glacial (6.10001.2500, 64-19-7) Hydrochloric acid 32% (1.00319.2511) Sulfuric acid 98% (6.10276.2500)	Merck, Germany
Ammonium hydroxide (221228) NADase (E.C. 3.2.2.5, N-9879)	Sigma Chemical Co., 6050 Spruce St, St Louis, MO, USA
PIC A reagent (WAT085101, 6110102781)	Waters Corporation, Milford, USA

Note Description presented as product number, batch or lot number

Table 6.6 Details of chemicals used in the study of acrylamide

Chemicals	Supplier
Sodium thiosulphate(D3247, 103154)	Ajax Chemicals International Pty. Ltd., Level 24, 270 Pitt St, Sydney, NSW, Australia
Hydrobromic acid (48% solution) (254, 09322705024473)	Asia Pacific Speciality Chemicals Limited
Methanol (67-56-1, FCFM1H)	Burdick and Jackson, USA
1-chloronapthalene (27719, 1808370)	BDH Chemicals, Australia Pty. Ltd., 207 Colchester Rd, Kilsyth, VIC, Australia
Ethyl acetate (1365968722) *n*-hexane (6.10444.2500, 1268) Potassium bromide (K40889205013, 1.04905.0500) Sodium sulphate (anhydrous) (7757-82-6, 6.10264.0500)	Merck, Germany
Bromine (141199.2208, 0000068758)	Panreac Quimica SAU, Barcelona
Acrylaway ®3500 BG (H0100010, F102-2)	Novozymes, Denmark
Acrylamide for electrophoresis (>99%) (A3553, 029K0739)	Sigma Chemical Co., 6050 Spruce St, St Louis, MO, USA
2,3-dibromopropionamide (D1378, F1N01)	Trade TCI Mark, Tokyo, Japan

Note Description presented as product number, batch or lot number

Table 6.7 Description of flour sample used for this study

Description	Supplier
Baker flour Batch no. B9 P6	Manildra Group, Australia

Table 6.8 Label information of commercial instant noodles

Brand name	Ingredients (as listed)	Country of origin
Mama	Wheat flour, palm oil, salt	Thailand
Maggi	Wheat flour, tapioca starch, vegetable oil (antioxidant (320)), salt, vegetable gum (guar), mineral salts (501, 451, 500, 452), minerals (iron, zinc), vitamins (niacin, thiamin, riboflavin, folic acid)	Malaysia
MR. KON Noodles	Wheat flour, refined palm oil, starch	China
Doll instant noodle	Wheat flour, palm oil, salt, acidity regulator (501, 500, 339), emulsifier (322), Colour (100), antioxidant (306)	Hong Kong
Nissin	Wheat flour, palm oil (contain antioxidant 306), salt, mineral salt (501, 500)	Hong Kong
Megah Mee EE Fu Noodles	Wheat flour, salt, sodium carbonate, egg, cooking oil (RBD palm olien), water and artificial colour (yellow No5 Tartrazine)	Malaysia
Megah Mee Claypot Ee Mee	Wheat flour, salt, sodium carbonate, egg, cooking oil (RBD palm olien), water and artificial colour (yellow No5 Tartrazine)	Malaysia
Nong shim	Wheat flour (cereal containing gluten), palm oil, starch, salt, acidity regulator (potassium carbonate)	Korea
Indomie	Wheat flour, edible vegetable oil, salt, acidity regulator, stabiliser, mineral (zinc), tartrazine E 102	Indonesia
Myojo	Wheat flour (78%), palm oil (antioxidants [320, 306]), salt, acidity regulators (501, 500, 450)	Singapore
Otogi	Wheat flour, palm oil, modified potato starch, salt	Korea

Notes Additive code numbers (Food Standards ANZ, 2010b) correspond to:

Acidity regulator (339, 450, 500, 501): sodium phosphate (dibasic), potassium pyrophosphate, sodium bicarbonate, potassium bicarbonate (respectively)

Antioxidant (306, 320): tocopherols concentrate, mixed, butylated hydroxyanisole (respectively)

Colour (100): curcumin or turmeric

Emulsifier (322): lecithin

Mineral salt (451, 452, 500, 501): potassium tripolyphosphate, potassium polymetaphosphate, sodium carbonate, potassium carbonate (respectively)

Table 6.9 Description of equipment and instrumentation

Equipment	Manufacturer/supplier	Model no
Autoclave	SILTEX Pty Ltd, Australia	Siltex 250D & 250DV
BondElut®SCX	Varian Inc, USA	Sorbent Lot#: 1712707 Raw Silica Lot#: 0025006
Centrifuge[a]	Rowe Scientific Pty Ltd, Western Australia	Mistral 1000
Centrifuge[b]	Beckman Instrument, Inc, Germany	GS-15R, 360904. series 95H13
Magnetic hot plate	Industrial Equipment & Control Pty Ltd, Australia	CS76083V
Minolta Chroma Meter	Minolta Camera Co Ltd, Japan	CR 300
Oven	Contherm Designer Series, New Zealand	I01265
pH meter	Hanna Instruments, USA	pH 211
Sep-Pak® Vac RC (500mg) C18 cartidges	Waters Co, Ireland	Part No: WAT036945 Lot No: 028237110A
SPME Fiber Assembly 1cm, 23 GA, Manual 60 µm, CW (PEG) Purple	Supelco, USA	Lot: P383172
UV-visible Spectrophotometer (double beam)	Varian Inc, USA	Cary EL96053135
Ultra-Turrax homogeniser	Janke and Kunkel, Stanfen, Germany	T25
Vacuum manifold system	J.T. Baker Inc, USA	ENVI^{TM}
Water activity meter	Axair Ltd., Switzerland	Novasina ms1-aw

Note Centrifuge[a] used in niacin experiments; Centrifuge[b] used in acrylamide experiments

Table 6.10 Description items used in HPLC analysis of niacin

Equipment	Manufacturer/supplier	Model No
HPLC system	Waters Associates Inc, Milford, USA	M-6000A, SDS-7181
Lichrosphere®100 Reverse phase-18 (5µm) Column	Merck, Germany	824232, L321017
Nylon membrane filters	Magna, USA	1229639, 284957
Syringe	SGE, Australia	005312, E12-A-852
Syringe filter	Filtamate Separation Technologies. Labquip Technologies	FM13410T
UV-VIS spectrophotometric detector	Shimadzu Corporation, Japan	SPD-10AV, C20342800471
Vial	Altech Associates Australia	95191

Table 6.11 Description items used in GC-MS analysis of acrylamide

Equipment	Manufacturer/supplier	Model No
GC-MS	Agilent Technologies, Delaware, USA	5973 Serial no. US00030656
Auto sampler	Agilent Technologies, Delaware, USA	G2614A Serial no. US12311837
BPx5 column (30 m × 250 µm i.d., 0.25 µm film thickness)	SGE, Australia	10244C20

Table 6.12 Description items used in GC-FID analysis of acrylamide

Equipment	Manufacturer/supplier	Model No
GC-FID	Shimadzu Corporation, Japan	GC-17A system
Solgelwax column (30m × 250 µm i.d., 0.25 µm film thickness)	SGE, Australia	9498E07

Table 6.13 Description items used in GC-ECD analysis of acrylamide

Equipment	Manufacturer/supplier	Model No
GC-µECD Detector	Agilent Technologies, Delaware, USA	6890 Serial no. US00027194
Auto sampler	A Total Analytical Solution (ATAS), FOCUS	SNO. 121666
Data handling system	Agilent GC Chemstation	Rev B04.01
DB-WAX Column (15m × 0.320 mm i.d., 0.50 µm film thickness)	Agilent Technologies, Delaware, USA	US7174527B

6.3 Preparation of instant noodles on a laboratory scale

Ingredients used for instant noodles

The primary ingredients used to make instant noodles were adopted from Moss, Gore and Murray (1987). This experiment used 300 g wheat flour per batch, 105 g water, 0.6 g salt and 0.60 g of kansui (alkaline salts). For the last of these, a mixture of 0.36 g potassium carbonate and 0.24 g sodium carbonate was used and palm oil was selected for deep frying. The specifications of equipment applied for noodle preparation are presented in Table 6.14. The major pieces of equipment used in the laboratory for noodle preparation are shown in Figure 6.1.

Table 6.14 Description of equipment and instrumentation used for making instant noodles on a laboratory scale

Equipment	Manufacturer/supplier	Model no
Cutting attachment for noodle maker	Imperia, Torino, Italy	MOD 150
Deep fryer[a]	Roband Woodson, Australia	10717
Deep fryer[b]	Sunbeam, China	DF4400, Type 529
Kenwood mixer	Kenwood Ltd, Britain	KM210, Serial no. 0309397
Noodle maker	Imperia, Italy	Domestic 'spaghetti machine' MOD 150, design no. 1048534
Oven	Memmert, GmbH, Germany	Type: UML 500, F No: 891319, NIN 12880-KI
Steamer	Sunbeam, China	RC2610, Type 638
Digital thermometer	Hanna, Romania	HI93503

Notes 1 Deep fryer[a] used in niacin experiments
2 Deep fryer[b] used in acrylamide experiments

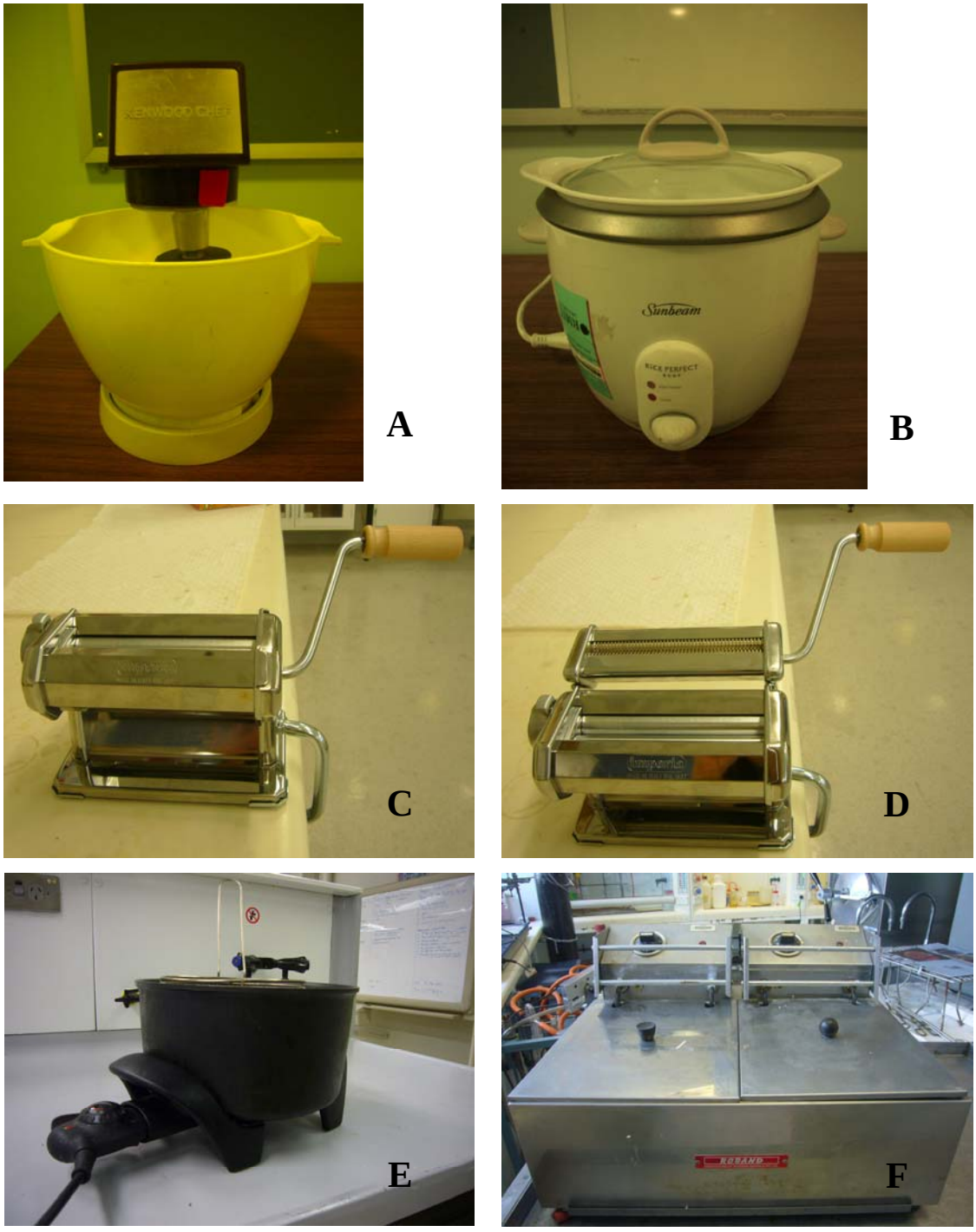


Figure 6.1 The equipment used in making instant noodles on a laboratory scale

A: Mixer, B: Steamer, C: Noodle maker, D: Noodle maker with cutting attachment, E: Deep fryer used in niacin experiments, F: Deep fryer used in acrylamide experiments

Processing of instant noodles

The procedure for making instant noodles on a laboratory scale followed the typical procedure outlined in the literature review (Chapter 4, Section 4.4) in terms of the basic principles. The primary steps for making instant noodles involved mixing raw materials, resting the crumbly dough, sheeting the dough into two dough sheets, compounding two sheets into one, sheeting the dough piece repeatedly to give the specified thickness and cutting into noodle strands (Hou & Kruk, 1998).

Mixing: All ingredients except flour were predissolved in the distilled water. Salt water was added into the mixing bowl (Kim, 1996). Mixing time was one min at speed one, and then mixing was suspended briefly so that all the flour and dough pieces could be scraped down from the side of the bowl. Next, mixing was continued for one min at speed one, followed by four min at speed four. Then the dough was rested at room temperature for 30 min.

Rolling: The dough crumbs were combined into a ball by hand manipulation and the resultant dough was passed to the noodle maker. This was passed through the rolls eight times with a decreasing gap applied at each successive pass until the desired thickness was achieved. The gap between the finishing rolls at the completion of this process was setting. Then, the cutting attachment was used to cut the sheet into the strands.

Steaming: Fresh noodle strands were placed in a steamer and steamed over vigorously boiling water for 2 min. Then, they were removed from the steamer and placed onto dry paper towel for 30 s in order to drain the excess water.

Frying: The noodles from the steaming step were then immediately placed into a deep fryer with palm oil that had been preheated to 150°C. Noodles were routinely fried for 45 s. Frying time and temperature were varied during the experiments on acrylamide formation (described in Chapter 10).

Draining and cooling: The fried noodles were removed from the oil using a wire basket and allowed to drain for 30 s. Noodles were then transferred to absorbent paper and allowed to cool at room temperature for 20 min prior to placing into a sealed bag for storage.

6.4 General methods for characterisation of flours and noodle samples

In the analysis of all samples at least duplicate measurements of individual samples were assessed statistically and are reported as the mean value ± standard deviation.

6.4.1 Moisture determination

The moisture contents of noodle samples were measured following the air oven method (AACC, 1995a) and analysis of each sample analyses was carried out in duplicate. Empty aluminum moisture dishes with lids were firstly placed into a pre-heated oven set at 130 ± 3°C for one h. The empty dishes were taken out of the oven and cooled in a desiccator containing active silica gel desiccant for 20 min and then weighed. A sample of approximately 2 g was to accurately weigh into each pre-weighed. Then these were covered dished were placed into the oven with the corresponding lid placed under the respective dish and dried at 130 ± 3°C for one h until a constant weight was attained. The loss in weight was used calculate the moisture content of the samples using the following equation:

$$\text{Moisture content (\%)} = \frac{\text{Loss in weight of dish, lid and sample upon drying}}{\text{Initial weight of sample}} \times 100$$

6.4.2 Measurement of the pH of noodle samples

The pH values of flour and noodle samples were determined by the AOAC procedure (AOAC, 1990a). Samples of approximately 10 g were blended thoroughly in 100 mL of distilled water using the Ultra-Turrax homogeniser. The mixture was allowed to settle for approximately 30 min after which the supernatant liquid was decanted. This liquid was tested with a calibrated pH meter and the analysis was carried out in triplicate.

6.4.3 Measurement of the instant noodle colour

The instrument used to measure instant noodles was a Minolta Chroma Meter (model CR-100) equipped with a 50 mm diameter measuring head (Grant, Doehlert, McMullen & Vignaux, 2004). The instrument was first calibrated using the white calibration tile supplied by the manufacturer. Colour scores were obtained for *L**, *a** and *b**. The *L**

value measures the degree of whiteness, the a* value measures the degree of hue and the b* value measures the degree of yellowness (Pomeranz & Meloan, 1994).

6.4.4 Measurement of water activity of instant noodle samples

The measurement of water activity for each sample was carried out using the water activity meter (see Table 6.9 for details). Approximately 5 g of noodle sample was placed into the plastic container provided and this was transferred to the measuring chamber of the instrument. A period of approximately 30 min was allowed until the reading became stable and the values for water activity were recorded in triplicate.

6.5 Procedures and calculations applied in the analysis of niacin

6.5.1 Procedures used in the validation of vitamin analysis methods

A variety of approaches were used to ensure the validity of the methods and the result obtained. During the validation of methods, the initial approach was to measure standard solutions of niacin. Secondly, the procedure involved recovery studies in which noodle samples were spiked with appropriate amounts of the standard vitamin compound prior to extraction. Recoveries were calculated as follows:

$$\text{Recovery (\%)} = \frac{\text{(vitamin in spiked sample - vitamin in unspiked sample)}}{\text{vitamin added in spiked sample}} \times 100$$

6.5.2 Calculation of niacin content using HPLC

The niacin content was calculated from the standard curve. At the start of a series of HPLC measurements, at least replicate analyses of the standard solution were performed and the average peak areas were calculated. Then, these were compared with those of the sample peak areas. For this comparison, the weighed portion, the amount of aliquot used and the dilutions were taken into account.

6.5.3 Preparation of analytical curve

The peak areas of standard solutions was used directly and plotted using the scatter option in the Microsoft® Excel with concentration of the individual vitamin (µg/mL) on the x axis and the corresponding peak areas on the y axis. A linear regression equation of the form (y = mx + c) gave the equation and the R^2 value was also recorded. The latter value was considered and the analyses were repeated if this value was lower than 0.95.

6.5.4 Calculation of niacin content

The average peak areas for each sample tested were used in the calculation of niacin concentration using the linear equations. The dilution factor and the original sample weight used during the sample preparation and SPE were applied. The following equation was used to calculate the experimental value:

$$\text{Niacin content (mg/100g)} = T \times \frac{\text{Dilution factor}}{W} \times 100$$

Where

T = The concentration of niacin content calculated using the calibration equation (expressed in µg/mL)

W = amount of sample originally weighed (expressed in g)

100 = conversion factor so that result is expressed per 100 g of sample

After determination of individual niacin contents, the data were expressed on a dry weight basis. The purpose was to facilitate the direct comparison of the results particularly for different sample types. All samples analysed for niacin were also tested for moisture content and the following general equation was applied:

$$\text{Niacin content (adjusted to a constant moisture basis)} = \text{niacin content (as is basis)} \times \frac{100 - \text{constant moisture}}{100 - \text{actual moisture of sample}}$$

In all cases the data were recalculated to a dry weight basis (where the constant moisture figure is zero) so the equation was used in the form:

$$\text{Niacin content (adjusted to a dry basis)} = \text{niacin content (as is basis)} \times \frac{100}{100 - \text{actual moisture of sample}}$$

6.5.5 Duplicate analyses and presentation of analytical results for niacin contents

In the analysis of samples for niacin content, at least duplicate sub-samples of each sample were extracted and multiple analyses were also performed on each extract obtained. The results of replicate analyses of each sample have been calculated and presented as mean value ± sd. In order to ensure the validity of analytical results a sample of the breakfast cereal was used as a reference material (compare with Table 6.3) and this was analysed within each batch of samples tested.

6.6 Preparation of solutions used in the study of niacin

Reagent solutions

Hydrochloric acid solution (0.1M): 4 mL of 37% HCl was diluted with milliQ water to make 500 mL of solution.

Sodium hydroxide solution (5.0M): 20 g of NaOH was dissolved in 100 mL milliQ water.

Sodium hydroxide solution (7.5M): 3 g of NaOH was dissolved in 10 mL milliQ water.

Sulfuric acid solution (2.0M): 10.7 mL of concentrated H_2SO_4 was diluted with milliQ water to make 100 mL of solution.

Sulfuric acid solution (2.0 N): 53.5 mL of H_2SO_4 was dissolved with 500 mL of milliQ water.

Ammonium hydroxide in methanol (2%): 1 mL of concentrated ammonium hydroxide solution was diluted with methanol to make 50 mL of solution.

NADase solution: enzyme preparation (3.0 mL) was mixed with 5 mL of 100mM phosphate buffer (pH 6.8).

Sodium acetate-acetic acid buffer pH5.6 (0.5M): 45.5 mL of 0.5M sodium acetate was mixed with 4.5 mL of 0.5M acetic acid.

Standard solutions

Once prepared, these solutions were stored for approximately 4 weeks in the refrigerator.

Nicotinic acid stock solution (1 mg/mL): 100 mg of nicotinic acid was dissolved and made up to 100 mL with milliQ water.

Nicotinic acid working solution (100 µg/mL): stock standard solution was diluted ten fold with milliQ water.

Nicotinamide stock solution (1 mg/mL): 100 mg of nicotinamide was dissolved in mixed well and made up to 100 mL with milliQ water.

Nicotinamide working solution (100 µg/mL): stock standard solution was diluted ten fold with milliQ water.

6.7 Method of HPLC analysis for analysis of niacin contents (Juraja et al., 2003)

Sample test solutions were analysed using HPLC in conjunction with a UV-VIS spectrophotometric detector. The separation was performed on Lichrosphere®100 reversed-phase C18 column, length 125 × 4.0 mm, 5 µm pore size. The mobile phase consisted of 15% methanol and 85% milliQ water containing 0.005M PICA reagent and the flow rate of the mobile phase was 1.2 mL/min. Nicotinic acid was detected at 254 nm and the total HPLC run time required for each analysis was set to 10 min. Chromatograms were recorded using the HP3395 integrator (Hewlett Packard) which provided retention times for each peak along with peak areas. For the purpose of quantitation of niacin contents, standard solutions were injected with each batch of sample extracts analysed. The calibration and stability of the detector were monitored at the beginning of each set of samples as well as during the series of analyses.

6.7.1 Evaluation of procedures used in preliminary extraction niacin contents by HPLC

Method of Juraja et al. (2003)

The extraction for this method involved homogenising sample (1 g of sample) with 0.75 g of calcium hydroxide and 20 mL of milliQ water followed by autoclaving at 121°C for 2 h (approximately 104 kPa). The homogenate was then diluted to approximately 50 mL using milliQ water taking care that the volume did not exceed 50 mL, mixed thoroughly and allowed to cool. The final volume was adjusted to 50 mL with milliQ

water and then centrifuged at 0°C and 2500 rpm for 15 min. A 15 mL aliquot of the supernatant was adjusted to pH 7 using aqueous oxalic acid (10% followed by 1% as the pH approached 7) and made up to 25 mL with milliQ water. The suspension was then centrifuged at 2500 rpm for 10 min to precipitate the calcium oxalate. A 500 mg C18 Sep-Pak Vac column and a 500 mg SCX column were connected in series and conditioned with 10 mL of methanol and 10 mL of milliQ water. A 10 mL aliquot of the supernatant was loaded onto the C18 column and washed with 5 mL milliQ water. Then the C18 column was discarded and the SCX column was washed with 5 mL of methanol. Nicotinic acid was eluted from the SCX column with 5 mL of a freshly prepared 2% solution of ammonium hydroxide in methanol. The solvent was evaporated to dryness under a stream of nitrogen at room temperature and the re-dissolved in 1 mL of milliQ water. The solutions were filtered through 0.45 µm filter discs.

Method of Lahély et al. (1999)

Finely samples (5 g) were extracted with 30 mL of 0.1M HCl and then heated in a water bath at 100°C for one h. After being allowed to cool, the solution was made up to 50 mL with milliQ water and centrifuged at 1250 rpm for 10 min to facilitate separation of the precipitate and the supernatant. A 25 mL aliquot of the latter was added to 5 mL of a 5M NaOH solution and then autoclaved at 120°C for one h. After cooling, the pH was adjusted to 4.5 and the solution made up to 50 mL with milliQ water. The solution then was filtered through a 0.45 µm filter and analysed using HPLC.

Method of Windahl et al. (1999)

Approximately 1 g of sample was mixed with in 10 mL of 2M H_2SO_4 and the volume adjusted to 25 mL with milliQ water. The solution was again mixed thoroughly and autoclaved for 2 h at 121°C (104 kPa) and allowed to cool. Then, the mixture was diluted to 50 mL with milliQ water and centrifuged at 2500 rpm for 15 min at 0°C. A 15 mL aliquot was adjusted pH to 7 with saturated barium hydroxide solution and made up to 100 mL with milliQ water. The resultant suspension was centrifuged at 2500 rpm for 10 min at 0°C. A 500 mg of C18 Sep-Pak Vac cartridge and a 500 mg SCX were connected together and conditioned with 10 mL of methanol and 10 mL of milliQ water. A 20 mL aliquot of the supernatant was loaded onto the combined columns, after which the C18 cartridge was disconnected and discarded. Then the SCX column was washed with 5 mL of methanol and the nicotinic acid was recovered from the SCX

column using 5 mL of freshly prepared 2% solution of NH_4OH in methanol. The solvent was evaporated to dryness under a stream of nitrogen at room temperature and the residue was re-dissolved with 1 mL of milliQ water for HPLC analysis.

Method of Ndaw et al. (2002)

A finely ground sample (5 g) was extracted with 50 mL of 100mM phosphate buffer and 200 µL of NADase solution. The mixture was incubated at 37°C for 18 h and then the solution was made up to 100 mL with milliQ water in a volumetric flask. The solutions were filtered through a filter paper and then through a 0.45 µm filter.

Method of LaCroix and Wolf (2001, 2002, 2007)

Approximately 2 g of sample was extracted with 2 mL of 2.5N H_2SO_4 by autoclaving at 121°C for 45 min. The acid digest was adjusted to pH 6.5 with 7.5M NaOH and immediately readjusted to pH 1.0 with 2.5N H_2SO_4. Then, the filtrate was adjusted to 35 mL with milliQ water. A SCX cartridge was prewashed with three 6 mL portions of methanol, followed by three 6.0 mL portions of milliQ water. Homogenate (3 mL) was passed through the cartridge and then flushed with 3 mL of milliQ water. Nicotinic acid was eluted with two 6 mL portions of 0.5M sodium acetate-acetic acid (pH 5.6). Then the final volume was adjusted to 15 mL with 0.5M sodium acetate-acetic acid buffer prior to analysis by HPLC.

6.8 Preparation of reagents used in the study of acrylamide

The saturated bromine-water solution: 8 mL of bromine was dissolved in 500 mL milliQ water until precipitation became visible.

Sodium thiosulfate solution (1M): 12.40 g was dissolved in milliQ water and then diluted to 50 mL.

Potassium bromide and sodium sulfate anhydrous: calcinated in muffle furnace 600°C for 4 h and stored in tightly closed containers at room temperature.

Preparation of standard solution

Acrylamide was used to prepare standard solutions as follows:

Stock solution (500 µg/mL): Acrylamide (0.05 g) was weighed and then dissolved and diluted to 100 mL using methanol.

Working solution: 2 mL of stock solution was diluted to 100 mL with milliQ water to give a concentration of 10 µg/mL.

2,3-DBPA was used to prepare standard solutions as follows:
Stock solution (500 µg/mL): 2,3-DBPA (0.05 g) was weighed and then dissolved and diluted to 100 mL using ethyl acetate
Working solution: 1 mL of stock solution was diluted to 100 mL with ethyl acetate to give a concentration of 5 µg/mL

6.9 Procedures used in the analysis of acrylamide in instant noodles

Method of Zhang et al. (2006)

Sample extracts were injected into a Hewlett Packard GC system 5973 fitted with a Mass spectrometric detector. A BPx5 column was used (length 30 m × ID 0.25 mm × film thickness 0.25 µm). The carrier gas was Helium flowing at a rate of 1 mL/min. The column was held at 40°C for 3 min then programmed to increase at a rate of 20°C/min to 250°C and then held for 5 min at 250°C. The injections were carried out in splitless mode with the temperature of 280°C. Under these conditions, ions monitored were *m/z* 70, 149, and 151 for 2-bromopropenamide.

Method of Lee et al. (2007)

Samples of approximately 10 g were extracted with 100 mL of milliQ water at room temperature by supersonic nebuliser for 30 min following by centrifugation at 5000 rpm for 10 min. After that a 1.5 mL aliquot was diluted with 15 mL milliQ water and then mixed with 15 mL phosphate buffer solution (pH 7). The SPME fiber was immersed directly in the mixed solution to extract acrylamide at room temperature for 20 min. After this the fibre was removed from the solution and transferred directly into the injector assembly of the GC system, in this case into a Shimadzu GC-17A system with FID system. The absorbed acrylamide was released from the fibre and chromatographed on a Solgelwax column (30 m length × ID 250 µm × 0.25 µm film thickness) using hydrogen as the carrier gas at a flow rate of 1.5 mL/min. The injection was performed in the spiltless mode for one min with an injector temperature of 220°C. The oven temperature was set at 50°C and held for one min followed by a linear temperature gradient of 5°C/min up to 220°C.

Procedures based on Zhu et al. 2008

Sample preparation

Noodle samples (approximately 10 g) were homogenised with approximately 80 mL of milliQ water using the Ultra-Turrax. Four aliquots were weighed into four 100 mL volumetric flasks. Aliquots of 0, 0.5, 1.0 and 2.0 mL of acrylamide working solution (10 µg/mL) were added to the four flasks respectively. Then, each was made up with water to 100 mL and mixed by magnetic stirrer for 30 min, and then centrifuged at 3000 rpm for 15 min. A 25 mL of aliquot was taken into separatory funnel and 25 mL of *n*-hexane was added, mixing for 2 min. The aqueous phase was transferred into a 100 mL Erlenmeyer flask. Calcinated potassium bromide was dissolved with stirring and the pH adjusted between 1 and 3 by HBr (48%). Then 8 mL of saturated bromine-water solution was added prior to transfer into an ice bath in the dark for 1 h. After this, a few drops of 1M sodium thiosulfate solution were added until the yellow colour disappeared. The mixture was extracted with 25 mL ethyl acetate by shaking for 1 min. The organic phase was taken and dried over Na_2SO_4 before filtering through a 0.45 µm microfilter into an autosampler vial for GC analysis

GC-ECD analysis

Gas chromatography was performed on an Agilent 6890 system (Agilent Technologies, U.S.A.), equipped with a splitless injector and coupled to an electron-capture detector (ECD). This incorporated an Agilent microECD with a split/splitless injector. Sample extracts of 1 µL were injected at 60°C on a DB-WAX column (15 m × 0.320 mm i.d., 0.50 µm film thickness). The oven temperature was set to 60°C for one min and was increased to 240°C at the rate of 20°C/min. Carrier gas was nitrogen (constant flow at 1.0 mL/min). Chromatograms were recorded using Agilent Chemstation software.

6.10 Calculation and presentation of the analytical results of acrylamide content

Acrylamide content of the analysis sample was calculated using the ratio between average peak areas of internal standard and samples. A set value for the ratio of peak areas obtained for each sample were plotted as y-axis and concentrations of acrylamide added in the portions (0, 0.05, 0.1 and 0.2 µg/mL) were plotted on the x-axis. The absolute value for the x-axis obtained from the calibration curve was calculated as the

amount of acrylamide in these unspiked portion of samples when the value of the y-axis was equal to zero. For the final calculation, the weighed portion, the amount of aliquot used and the dilutions were taken into account.

$$\text{Acrylamide content (µg/kg)} = T \times \frac{\text{Dilution factor}}{W} \times 1000$$

Where

T = The concentration of acrylamide calculated using the calibration equation (expressed in µg/mL**)**

W = amount of sample originally weighed (expressed in g)

1000 = Conversion factor so that result is expressed per kg of sample

At least triplicate analyses were performed and the confidence interval of a series of values was also calculated. These calculations were carried out using Microsoft® Excel.

6.11 Statistical analysis

One way analysis of variance and Tukey's test were applied using Minitab (Minitab Inc, USA), version 15.

Chapter 7

Results and discussion: Preliminary assessment of procedures for extraction and quantitation of niacin from cereal-based foods

The purpose of this chapter is to describe and discuss the results obtained during the initial evaluation of procedures for extraction and measurement of niacin in cereal-based foods.

7.1 Introduction

From the literature review relating to niacin analysis procedures presented in Chapter 2, a wide range of methods have previously been applied to measurement of niacin in cereal-based foods. It has been reported that various problems have been encountered and these include the over estimation of niacin contents, the time-consuming nature of many of the procedures as well as the use of hazardous chemicals in some cases. Since instrumentation for chromatographic analysis became widely available, HPLC has been used for measurement of niacin in foods including those prepared from cereal flours. This has overcome the problems associated with the use of hydrogen cyanide associated with some of the traditional procedures. In addition, a wide variety of approaches to sample extraction and treatment of the extracts have previously been investigated and these include the use of either acid or alkaline reagents as well as sequential treatments with both of these.

7.2 Evaluation of alternative HPLC methods for niacin analysis

Due to the reports that HPLC provides a practical and safe approach to the analysis of niacin compounds in foods, this was selected for evaluation in the current study. Initially this was evaluated using solutions of nicotinic acid and nicotinamide prepared as standards, in conjunction with spectrophotometric detection. The first objective of these experiments was to establish suitable chromatographic conditions that would allow the determination of niacin in sample extracts.

The HPLC conditions for the first trial were adopted from those reported by Lahély et al. (1999), using a reversed-phase C-18 column and the mobile phase consisted of 0.07M potassium dihydrogen phosphate with a flow rate of 1.2 mL/min. Despite repeated attempts, when this was trialled, no peaks could be detected for either the nicotinic acid or the nicotinamide standard solutions.

As an alternative, a gradient elution approach was adopted with modification from a more recent investigation in which folic acid was measured in a noodle matrix, similar to that to be analysed in the current study (Hau Fung Cheung et al., 2008). Again a reverse-phase C-18 column was used and the gradient involved phase A containing 1% of glacial acetic acid in milliQ water and phase B was acetronitrile. Whilst peaks were obtained, these were neither symmetrical nor sharp and attempts to enhance these attributes were not successful.

Following further preliminary work, the chromatographic procedures found to be useful for quantitation of niacin were those adopted from Juraja et al. (2003) and described more fully in Chapter 6 (Section 6.7). These represent an isocratic system, utilising HPLC with a reversed-phase C-18 column and a mobile phase consisting of 15% methanol and 85% milliQ water containing 0.005M PIC A reagent with a flow rate of 1.2 mL/min. The latter compound was incorporated as an ion pairing reagent in order to facilitate separation of nicotinamide and nicotinic acid and both were readily detected at 254 nm. The standard compounds eluted at retention times of approximately 2.2 and 3.9 min, respectively and examples of the resultant chromatograms are shown in Figures 7.1 and 7.2. These demonstrate the consistent observation of symmetrical and sharp peaks as well as effective separation of the two vitamers.

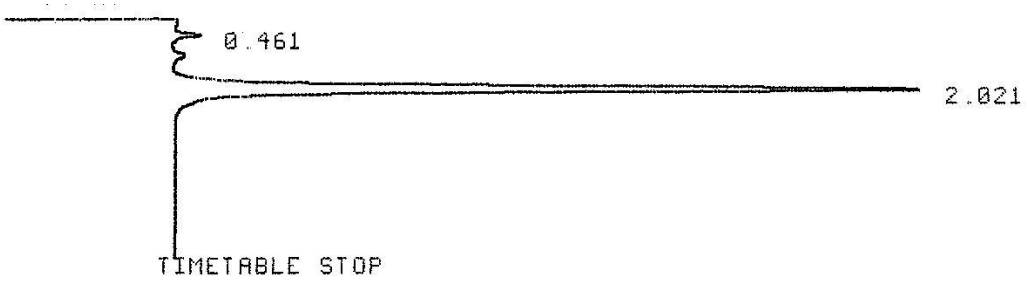


Figure 7.1 Chromatogram obtained for nicotinamide solution using the procedure of Juraja et al. (2003)

Note the concentration of the solution injected was 100 µg/mL, volume of injection was 20 µL

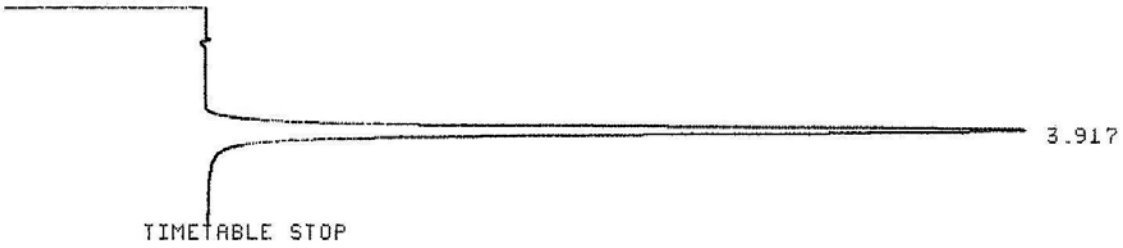


Figure 7.2 Chromatogram obtained for nicotinic acid solution based on the procedure of Juraja et al. (2003)

Note the concentration of the solution injected was 100 µg/mL , volume of injection was 20 µL

A series of standard curves were produced for quantitation of nicotinic acid and for this, standard solutions were generally prepared to cover the range of 20 to 100 µg/mL. The curves consistently had excellent linearity with high correlation coefficients and a typical example is presented in Figure 7.3. It was also often noted that minor differences in the equation of the lines were observed from day to day, possibly reflecting the variations in conditions including those of elution time and the ambient temperature conditions. Therefore to ensure reliability of the data a set of standard solutions was injected along with each batch of noodle extracts analysed during the study.

It is also noted that for some extraction methods trialled in this study, it was necessary to prepare standard curves for both nicotinic acid and nicotinamide, for example when enzymatic extraction was investigated. On the other hand, for most procedures it was sufficient to make a calibration for only nicotinic acid as the acidic/alkaline conditions employed result in the conversion of all of the nicotinamide present into nicotinic acid.

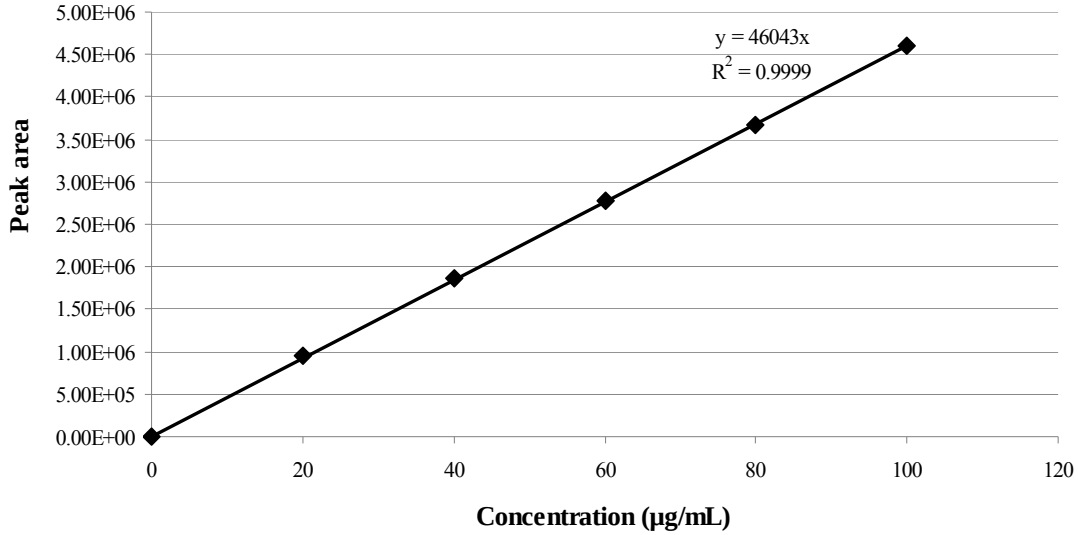


Figure 7.3 A typical standard curve obtained for nicotinic acid using the HPLC procedure of Juraja et al. (2003)

7.3 Evaluation of extraction procedures for niacin analysis

Following the establishment of a suitable approach for HPLC analysis of niacin compounds, extraction conditions were considered. Based upon previous observations on a number of vitamins, including niacin, it is recognised that a critical issue to the overall optimisation of procedures for the analysis is that of extraction. For niacin, various approaches have been published and these include the application of alkaline, acid and enzymatic extraction procedures.

For the preliminary evaluation of extraction, alkaline extraction was firstly set up, based on the previous work of Juraja et al. (2003). This utilises alkaline extraction in the presence of solid calcium hydroxide, in conjunction with the use of SPE cartridges to remove interfering components from extracts. The particular SPE materials trialled were C18 Sep-Pak Vac and SCX columns (500 mg in both cases). It is noted that during this study a variety of SPE procedures were trialled and in all cases the particular cartridges were used in accordance with instructions provided by the manufacturer. In particular, in the preparation of these materials, a slow flow rate of 1-2 drops per s was consistently applied, as well as throughout the subsequent loading of the sample extracts and

processes of elution and recovery of the niacin. This approach was designed to maximise recovery and hence enhance the reproducibility of results for the samples.

In order to facilitate these evaluations, a reference sample supplied by AACC International was used for validation purposes. This was supplied with results obtained by a number of laboratories for various components including niacin. When this sample was analysed using the extraction Juraja et al. (2003) in conjunction with the HPLC procedure evaluated and described here, the results obtained for the niacin level were consistently below those expected (Table 7.1). It was also found that when the same approach was applied to samples of wheat flour, no peaks for niacin could be detected in the resultant chromatograms. This indicates that either the levels of naturally occurring niacin were too low to be reliably measured or variations from SPE column and/or loss during solvent removal. Alternatively this extraction and clean-up approach is not suitable for detection of niacin at these levels in cereal food samples.

Table 7.1 Niacin results obtained for alkaline extraction and SPE treatment of reference sample

Description	AACC sample
Number of replicates	9
Mean value	10.60 mg/100g
Expected value	26.51 ± 3.73 mg/100g
Range of results	5.66-16.94 mg/100g
Standard deviation	4.65
Confidence interval	3.44

Note The expected value is expressed as mean ± standard deviation

In considering these observations and results obtained for the validation of the analysis, an additional step was taken to ensure the reliability of the standard solutions used in deriving the calibration data. For this checking, solutions were analysed by UV-visible spectrophotometry and the results compared with expected values based upon published molar extinction co-efficients. Solutions of nicotinic acid and nicotinamide were

prepared at known concentrations corresponding to 10 μg/mL and these were placed into quartz cuvettes. Using a double beam spectrophotometer the absorbances were recorded against those of a reference cell containing a blank of milliQ water and the resultant absorption spectrum for nicotinic acid is presented in Figure 7.4. This shows a relatively sharp peak with a maximum absorbance occurring at a wavelength of 261.5 nm. The spectrum obtained for a solution of nicotinamide was very similar in appearance to that shown for nicotinic acid.

The absorbance readings at the respective peaks were used in conjunction with published extinction coefficients originally reported by Hughs, Jellinek, and Ambrose (1949) and Jellinek and Wayne (1951). It is noted that the pH of the solutions was also measured as this had previously been shown to influence the extinction values for both compounds. The calculated data are compared in Table 7.2 and these confirm the purity and concentrations of both of the solutions, based on the measurement of absorbance at 261.5 nm.

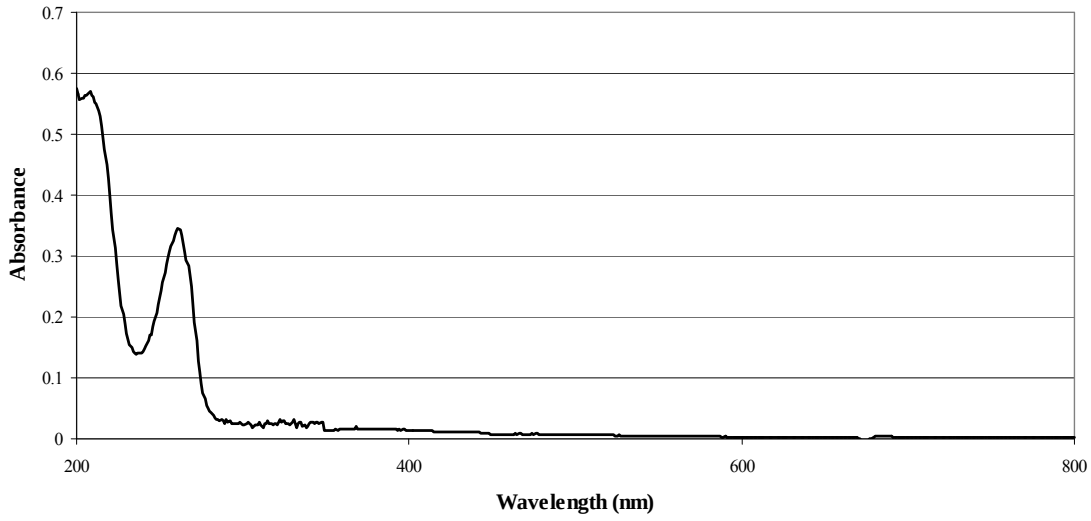


Figure 7.4 The absorption spectrum obtained for the standard solution prepared with nicotinic acid

Table 7.2 The UV absorbances of nicotinic acid and nicotinamide standard solutions

	$A_{261.5}$		pH of solution
	Observed	**Expected**	
Nicotinic acid	0.3460	0.3249	4.71
Nicotinamide	0.2410	0.2375	6.39

7.4 Further evaluation of SPE approaches in niacin analysis

The results of the preceding trials indicated that SPE has potential as a means of clean-up for sample extracts. On this basis, further investigations were pursued and in order to assess the retention of the vitamin molecules on the mini-columns as well as subsequent release, absorbance values of the solutions including eluates were recorded at each step using the UV-visible spectrophotometer set at 261.5 nm.

Initially, a 500 mg C18 Sep-Pak Vac column, as well as a 500 mg SCX column, were set up separately and each was preconditioned with 10 mL of methanol followed by 10 mL of milliQ water. A volume of 10 mL of 10 μg/mL of nicotinic acid was loaded onto each column followed by washing with 5 mL of methanol. Nicotinic acid was eluted from the SCX column with 5 mL of a 2% solution of ammonium hydroxide in methanol which was freshly prepared following the procedure described in Section 6.6. The eluate was collected into a preweighed container and absorbance measured. Then the solution was treated using a stream of nitrogen at room temperature in order to remove volatile materials which might have contributed to the absorbance of the solution. This resulted in the evaporation of some of the solvent, so that the resultant solution was diluted prior to measurement of the final absorbance. The results obtained are presented schematically in Table 7.3.

The absorbance data presented in Table 7.3 has been used in the calculation of the concentrations of nicotinic acid at each stage and the results are presented in Table 7.4. These demonstrate that the recovery of nicotinic acid eluted from the C18 Sep-Pak Vac

column appeared to be almost 100% whereas the apparent loss of nicotinic acid during use of the SCX column was in excess of 50%. It is noted that the apparent loss of nicotinic acid could be due to incomplete elution from the SCX column. In this experiment the concentrations and recoveries of the vitamin were measured spectrophotometrically. Despite the fact that highly purified standard nicotinic acid was used, this approach potentially lacks specificity because any other compounds present may absorb at the same wavelength of 261.5 nm. Accordingly, HPLC was further used to determine the actual amounts of nicotinic acid present of key stages and the results are summarised in Table 7.5. The HPLC analyses show that the recovery of nicotinic acid for the C18 Sep-Pak Vac column and the SCX column appeared to be approximately 75% and 65%, respectively, based upon the HPLC measurements. Therefore, further investigation of this system was set up and the sequential application of these two columns was evaluated.

Table 7.3 The absorbance and pH values of solutions from two SPE columns at each step of treatment of a nicotinic acid solution

	C18 Sep-Pak Vac column		SCX column	
	$A_{261.5}$	pH	$A_{261.5}$	pH
Addition of 10 mL methanol	0.1000 (eluate)	8.35	0.9553 (eluate)	1.65
⇩				
Add 10 mL milliQ water	0.0167 (eluate)	6.55	0.0642 (eluate)	4.37
⇩				
Addition of a nicotinic acid solution (10 mL of 10 μg/mL)	0.2167 (prior to adding to column)	6.71	0.2225 (prior to adding to column)	6.93
⇩	0.1881 (eluate)	5.55	0.0294 (eluate)	3.16
Wash with 5 mL water	0.0556 (eluate)	7.53	0.0391 (eluate)	4.04
⇩				
C18 Sep-Pak Vac column is discarded				
⇩				
Wash with 5 mL of methanol			0.0612 (eluate)	8.33
⇩				
Add 5 mL of a freshly prepared 2% solution of concentrated ammonium hydroxide in methanol	Before stream of N_2		0.5572 (eluate)	10.80
	After stream of N_2		0.9661 (eluate, after twofold dilution)	

Table 7.4 Concentrations of nicotinic acid and pH values of solutions from two SPE columns at each step of treatment of a nicotinic acid solution

	C18 Sep-Pak Vac column		SCX column	
	Concentration (µg/mL)	**pH**	**Concentration (µg/mL)**	**pH**
Add 10 mL of methanol				
⇩				
Add 10 mL milliQ water				
⇩				
Addition of a nicotinic acid solution (10 mL of 10 µg/mL)	9.23 (prior to adding to column)	6.71	9.10 (prior to adding to column)	6.93
⇩	7.47 (eluate)	5.55	0.70 (eluate)	3.16
Wash with 5 mL water	2.28 (eluate)*	7.53	1.07 (eluate)	4.04
⇩				
C18 Sep-Pak Vac column is discarded				
⇩				
Wash with 5 mL of methanol			2.51 (eluate)	8.33
⇩				
Add 5 mL of a freshly prepared 2% solution of concentrated ammonium hydroxide in methanol	Before stream of N_2		22.87 (eluate)	10.8
	After stream of N_2		11.43 (eluate)*	

Note * indicates the solutions and results which were used in the calculation of overall recoveries (Table 7.5)

Table 7.5 Concentrations and recoveries of nicotinic acid in final eluate solutions from two SPE columns of a nicotinic acid solution following analysis using HPLC

	Column	
	C18 Sep-Pak Vac	**SCX**
Concentration of final eluate (μg/mL)	7.56	16.70
Volume (mL)	9.99	3.88
Recovery (%)	75.0	64.8

7.5 Evaluation of alkaline extraction in conjunction with sequential SPE

In order to further assess the sequential SPE method described earlier, absorbances were measured at each step of the procedure involving alkaline extraction followed by SPE. Three sample combinations were trialled: AACC reference sample, the AACC sample spiked with 1 mL of 10 μg/mL nicotinic acid and spike only with 1 mL of 10 μg/mL nicotinic acid without sample (Tables 7.6 and 7.7). The results indicate that the absorbance values obtained are not consistent and some are much higher than those expected. This may be due to interference of other components extracted from samples, which co-eluate from SPE columns and have strong absorbance at 261.5 nm.

In order to more fully clarify the potential of this approach to extraction and sample treatment (Juraja et al., 2003), direct measurements of the recovery of niacin were made using HPLC and extracts prepared with and without spiking. Samples of wheat flour and the AACC reference sample were prepared and 1 mL of a 100 μg/mL nicotinic acid solution was added at the first step of extraction. The results of replicate trials are presented in Table 7.8.

Table 7.6 The absorbance and pH values of solutions at various stages of the process of alkaline extraction in the spiking study of the AACC reference sample

	Spiked without sample		**AACC sample with spike**		**AACC sample without spike**	
	$A_{261.5}$	**pH**	$A_{261.5}$	**pH**	$A_{261.5}$	**pH**
Mix sample with water (1g + 20mL)	3.8776	14.0	3.6317	14.0	2.2618	13.84
⇩						
Add $Ca(OH)_2$ (0.75 g)	0.4959	14.0	0.0243	14.0	0.2028	13.84
⇩						
Autoclave for 2 h then dilute to 50 mL	0.0297	13.95	0.0172	13.97	3.8566	13.81
⇩					0.2959 (1in10)	13.81
Subsample 15 mL		13.95	0.0856	13.97	3.8022	13.81
⇩					0.2799 (1in10)	13.81
Adjust pH to 7.0 using oxalic acid solutions		7.33		6.42		6.92
⇩						
Dilute to 25 mL	0.0697		0.0614		3.6273	
⇩					0.3761 (1in10)	
Take 10 mL and subject to SPE procedure			0.0089		3.5649	
⇩					0.3362 (1in10)	
Remove solvent, redissolve in 1 mL water, analyse by HPLC						

Table 7.7 The absorbance and pH values of eluate solutions from the SPE procedure of Juraja et al. (2003) following alkaline extraction (see Table 7.6)

	Spiked without sample		**AACC sample with spike**		**AACC sample without spike**	
	$A_{261.5}$	**pH**	$A_{261.5}$	**pH**	$A_{261.5}$	**pH**
Add 10 mL of methanol						
⇩						
Add 10 mL milliQ water						
⇩						
Add 10 mL aliquot of the supernatant	0.0331	3.48	0.0299	3.73	0.4581	2.17
⇩						
Wash with 5 mL water	0.0358 (eluate)	4.36	0.0278 (eluate)	4.20	0.4543 (eluate)	3.16
⇩						
C18 Sep-Pak Vac column discarded						
⇩						
Wash with 5 mL of methanol	0.0880 (eluate)	4.27	0.0711 (eluate)	4.60	0.1643 (eluate)	4.60
⇩						
Add 5 mL of a freshly prepared 2% solution of concentrated ammonium hydroxide in methanol	0.2688 (eluate) 1in1		0.3815 (eluate)		0.8581 (eluate)	

Table 7.8 Niacin results obtained for alkaline extraction

Description	Spiked without sample	AACC sample	Wheat flour
Number of replicates	4	4	8
Mean value (mg/100g)	2.14	10.94	3.16
Range of results (mg/100g)	1.91-2.32	9.14-12.8	1.45-5.32
Standard deviation	0.18	1.57	1.40
Confidence interval	0.18	1.54	0.97
Recovery (%)	257	56.7	527

From Table 7.8, the results indicate that the recovery of niacin was not consistent and some of the values were very much higher than expected. These observations may be due to the alkaline extraction releasing a large amount of those forms of niacin which are not bioavailable in foods, therefore providing a measure of "total niacin". In the case of the AACC sample, it is possible that some forms of niacin are not being extracted so that the values are under-estimated. Moreover a further problem encountered in the procedure was that the calcium hydroxide used in the extraction induces gelation of the starch, at least for the AACC sample. This may explain the observation as a factor contributing to the under-estimation of the true niacin value.

Again the data shows that the recoveries were not consistent and clearly interferences were occurring so that this alkaline extraction approach is not readily adaptable to the proposed studies of instant noodle samples. Thus to solve these problems, it was concluded that the use of alternative extraction solutions may have greater potential to provide reliable results when combined with SPE procedures.

7.6 Evaluation of a procedure involving only an acidic extractant

Acid extraction was further evaluated and the approach adopted was that of Windahl et al. (1999) and described in detail in Chapter 6 (Section 6.7.1). For this, the samples were extracted with 2M H_2SO_4 with heating in an autoclave for 2 h at 121°C followed by sequential SPE. The results obtained are presented in Table 7.9.

Table 7.9 Niacin results obtained for acid extraction of AACC reference sample

Description	AACC sample
Number of replicates	4
Expected values	26.51 ± 3.73 mg/100g
Mean value	4.49 mg/100g
Range of results	4.28-4.83 mg/100g
Standard deviation	0.24
Confidence interval	0.23

These results indicate that acid extraction give results substantially lower than those that were obtained originally by the laboratories contributing analytical data on the AACC reference sample. This probably reflects the release of nicotinamide from its coenzyme forms during acidic extraction with simultaneous hydrolysis to nicotinic acid whilst this treatment does not completely release all of the bound forms of niacin from cereal products (Windahl et al., 1999). These researchers compared acid and alkaline extraction for meat and fish samples: for these foods, acid extraction gave slightly higher levels whereas higher results were found for cereal products when alkaline extraction was applied. It was concluded that in order to release all of the bound niacin, alkaline extraction was required. Thus an acidic extraction appears to be suitable for non-cereal food products including meats and fish. However, Rose-Sallin, Blake, Genoud, and Tagliaferri (2001) reported that acid extraction with 0.1N HCl for one h at 100°C gave a similar niacin level to two-step extraction in a range of fortified foods and cereal products.

7.7 Evaluation of enzymatic extraction procedure

On the basis of the results obtained with the acid and alkaline procedures, alternative approaches were needed and hence it was decided to evaluate a procedure based on enzymatic extraction. For this, the work of Ndaw et al. (2002) was followed and applied to samples of wheat flour, breakfast cereals and AACC reference samples. The enzyme NAD glycohydrolase (NADase) from *Neurospora crassa* was selected for this study and the extraction was performed at pH 4.5 with incubation at 37°C for 18 h. The resultant chromatograms for the AACC sample showed two peaks which corresponded to nicotinic acid and nicotinamide. The results obtained for niacin are presented in Table 7.10.

Table 7.10 Niacin results obtained for enzymatic extraction

Sample	Nicotinic acid (µg/100g)	Nicotinamide (µg/100g)	Total niacin (µg/100g)	Expected niacin (mg/100g)
AACC	0.91 ± 0.91	1.87 ± 0.84	2.78	26.51 ± 3.73
Kellogg	0.78 ± 0.19	No peak obtained	0.78	8.3
Wheat flour	No peak obtained	No peak obtained	-	0.5-7.6

Notes 1 Results are expressed as mean ± standard deviation
2 The sources of the expected values were
AACC: data obtained by the AACC laboratories
Kellogg breakfast cereal: data provided in the nutrient information panel on the package of the product purchased commercially
Wheat flour: Scherz and Senser (2000); NUTTAB (2006)

The results indicate that the niacin values for each of the samples were below the range of results expected. It is known that NADase enzymes only hydrolyse bound forms of niacin (NAD and NADP) but they do not appear to catalyse any subsequent conversion of nicotinamide to nicotinic acid. Therefore this procedure also did not appear to be suitable for extraction of niacin in cereal-based food products. It is noted that in the original publication, Ndaw et al. (2002) proposed that this procedure was sufficient for extraction in foodstuffs containing large quantities of starch (including rice, wheat flour) or protein. They also indicated that, from their comparisons, the resultant nicotinic acid contents were slightly higher after acid extraction than enzymatic hydrolysis.

7.8 Evaluation of further procedures including acid/alkaline extraction

A further option presented in the literature is the procedure of Lahély et al. (1999) which involves hydrolysis using 0.1M HCl with incubation at 100°C for one h followed by hydrolysis with a 5.0M NaOH solution through autoclaving at 120°C for a further h. This was originally applied to the determination of niacin in a wide range of foods including beef liver, fruit juice, brewer's yeast, peanuts, tomato, biscuits, green peas, pork and veal. In order to further assess the suitability of this extraction procedure the approach was applied to wheat flour samples and the niacin content obtained was 48 mg/g which is very much higher than the expected range of 500 μg – 7.6 mg/100g (Scherz & Senser 2000; NUTTAB 2006). This is probably be due to the lack of any form of clean-up procedure in the published procedure applied here, following the extraction, so that interfering components from the food matrices are co eluting with niacin and absorbing at the wavelength used for detection.

Thus to validate this procedure, further evaluation was pursued by measuring the recovery of niacin with a spiking study. The wheat flour sample was spiked with 6 mg nicotinic acid in 1 g of flour sample at the beginning of procedure. The analytical results are provided in Table 7.11. For the spiked flour samples it was found that the recovery expressed as a percentage was particularly high. This is probably due to this method not employing a clean-up procedure after the extraction and the result is that niacin cannot be completely separated from the interfering components and a good recovery with high sensitivity cannot be obtained. Therefore, it was again concluded that this extraction is not appropriate for measuring niacin in cereal-based food products.

Table 7.11 Recovery of nicotinic acid from the flour sample spiked at a rate of 6 mg/1 g flour

	Nicotinic acid
Flour (mg)	48.00
Flour with spike (mg)	73.98
Calculated amount of spike (mg)	25.98
Recovery (%)	433

Further investigations of extraction were carried out using the alternative method described by LaCroix and Wolf (2007). Niacin was extracted with the acid digestion following by the use of SPE. This entailed autoclaving the sample at 121°C for 45 min with 2N H_2SO_4 and the SPE stage utilises a SCX column with recovery of the nicotinic acid using a 0.5M sodium acetate-acetic acid buffer of pH 5.6. The results obtained are presented in Table 7.12.

Table 7.12 Recovery of nicotinic acid obtained for acid extraction followed by SPE (LaCroix & Wolf, 2007)

	Before spiking	**Spiked at 132 µg/mL nicotinic acid**	**Recovery (%)**
Wheat flour (µg/mL)	No peak obtained	32.7	24.7
AACC Reference samples (µg/mL)	No peak obtained	No peak obtained	-

The results indicate that nicotinic acid could not be readily detected in the wheat flour and AACC reference samples. Moreover, the extent of recovery was quite low when the amount of spike corresponded to 3.3 mg nicotinic acid. The procedure did not appear suitable for extraction of either the flour or reference sample.

7.9 Evaluation of acid and alkaline extraction with SPE

In the context of the lack of useful results obtained using a variety of procedures another option was to combine two approaches that have been separately applied to sample extraction. Therefore, a modified extraction was further considered using a combination of acid and alkaline extraction described by Lahély et al. (1999) in conjunction with the SPE procedure reported more recently by LaCroix and Wolf (2007). This combination was evaluated and the results are summarised in Table 7.13. The analytical results gave mean recovery values that are very close to 100 %.

The validity of the procedure was further evaluated by measuring the recovery of niacin with a spiking study. The wheat flour sample chosen was used in preparation of noodles in later parts of this study. In analysing the spiked flour samples it was found that the recovery level of niacin was 76%. The alkaline treatment converts nicotinamide to nicotinic acid and liberates the nicotinic acid from its chemically bound form, whereas the acid treatment liberates nicotinamide from its coenzyme forms and simultaneously hydrolyses it to nicotinic acid. The two step extraction appears to have released only bioavailable forms of niacin while converting all vitamers to nicotinic acid (Rose-Sallin et al., 2001). Whilst this finally appeared to provide the basis of a valid approach to sample extraction, further investigation was needed to ensure the validity of the procedure especially for the samples of wheat flour products to be studied here.

Table 7.13 Recovery of nicotinic acid obtained for acid/alkaline extraction followed by SPE (LaCroix & Wolf, 2007; Lahély et al., 1999)

	Niacin (mg/100g)	**Recovery (%)**
Wheat flour	No peak obtained	-
AACC Reference sample	28.12 ± 4.4	107.96

Note Results are expressed as mean ± standard deviation

7.10 Further evaluation of solid phase extraction/clean up procedure

Earlier stages of this work indicated some variability and difficulties associated with the recovery of niacin following SPE procedures. Accordingly in an attempt to clarify the suitability of the SPE stage of the analysis, experiments were set up to observe the effect and significance of volumes of solutions used for elution from the SPE cartridges. These trials were based around the procedures described by Lahély et al. (1999) and LaCroix and Wolf (2007) with selected modifications. The sample selected for the comparison was the AACC reference material and the solutions originally suggested, including 0.1M HCl for extraction and 0.25M sodium acetate/acetic acid buffer for SPE were used in the current work. However, the volumes of these have been varied. Five gram samples were prepared by autoclaving at 121°C for 45 min in 30 mL of 0.1M HCl. After allowing these resultant solutions to cool, each was made up to 25 mL with milliQ water and filtered. A 20 mL aliquot was added to 5 mL of 5M NaOH solution and this was autoclaved at 120°C for one h. The pH of solution was adjusted to 1.0 and 3.0 mL of solution was passed through the SCX cartridge. The niacin was eluted using 3.0 mL aliquots of 0.25M sodium acetate-acetic acid buffer and this was done four times (that is 3.0 mL × 4 = 12.0 mL) rather than the single treatment involving 6 mL originally described and the results obtained are summarised in Table 7.14.

Table 7.14 Results for nicotinic acid content obtained following elution with successive volumes of 3 mL of 0.25M sodium acetate/acetic buffer

	Sequence of elution buffer used			
	First	**Second**	**Third**	**Fourth**
Nicotinic acid (mg/100g)	22.88 ± 6.77	No peak obtained	No peak obtained	No peak obtained

Note Results are expressed as mean ± standard deviation for duplicate trials

These data indicate that relatively consistent results were obtained when a single aliquot of 3 mL of 0.25M sodium acetate/acetic acid buffer was used for elution. Subsequent aliquots of eluent did not result in the further recovery of any detectable levels of nicotinic acid. The results calculated from the analyses of the eluates indicate that the average value for the content of the AACC reference sample was 22.88 mg/100 g (n=3).

This corresponds with a recovery of 86.3% in relation to the average result provided for the sample by AACC (n=3) and falls within the range of results originally obtained by the participating laboratories which analysed the sample. From the observations (Table 7.14), it is concluded that an appropriate and sufficient elution volume is 3 mL of 0.25M sodium acetate/acetic acid. This has the advantage for routine analysis that the reduced volume of eluate provides a correspondingly larger peak for reliable quantitation following HPLC.

Based upon these results this procedure appeared to show potential for determine nicotinic acid in AACC reference sample. The same analytical approach was then applied to a commercial breakfast cereal product and the results obtained are shown in Table 7.15. An example of a chromatogram is presented in Figure 7.5. The precision of the analyses was considered to be satisfactory and, in relation to the niacin level measured by the procedure, the value was almost twice that presented on the label by the manufacturer. This reflects the requirement under Australian regulations that claims cannot exceed 25% of the RDI value. In line with typical commercial practice it is highly likely that a significant "overage" was incorporated to ensure that the product at least meets the claimed levels even if losses have occurred during processing and storage.

Table 7.15 Niacin results obtained for a breakfast cereal sample

	Niacin content (mg/100g)	Label value (mg/100g)
Breakfast cereal	15.3 ± 1.8	8.3

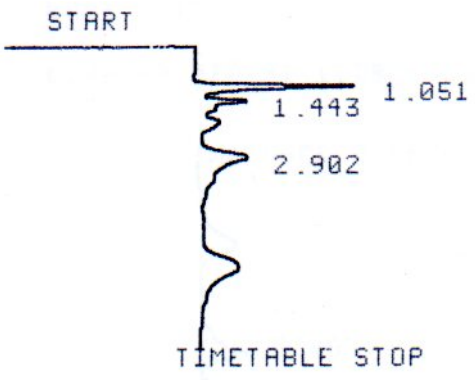


Figure 7.5 Chromatogram obtained using HPLC for breakfast cereal sample
Note Nicotinic acid has a retention time of 2.902 min

7.11 Summary of results obtained in the preliminary investigation of procedures for analysis of niacin

Extensive trials were carried out using published methods for extraction and analysis of niacin in cereal based foods. HPLC with UV-absorbance at 254 nm was used to analyse niacin and the resultant chromatograms gave a sharp peak corresponding to nicotinic acid. A number of different procedures were evaluated for the extraction of niacin components as well as extract treatment. Considerable challenges were encountered and many of the published methods were not effective for the samples of instant noodles. The use of either acid or alkaline extraction solutions of various concentrations did not give good recoveries. Published SPE procedures were also investigated. Ultimately the use of both acid and alkaline extraction solutions in a sequential extraction process, in combination with SPE using only a cation exchange cartridge, without a reversed phase mini-column, was found to give acceptable recoveries when trials of spiked extracts were applied. During this preliminary investigation, some cereal-based food materials were used for validation purposes and these included wheat flour, a fortified breakfast cereal and a reference sample.

Once a suitable method had been found that might be reliably utilised in a study of noodle samples, further studies were commenced on noodles prepared under laboratory conditions. Hence in the following chapter of this thesis, the validated analysis method has been used in further studies of the stability of niacin during processing of flour fortified with nicotinic acid for the manufacture of instant Asian noodles.

Chapter 8

Results and discussion: The measurement and stability of niacin in instant noodles prepared in the laboratory

The purpose of this chapter is to describe and discuss the results obtained for niacin during the analysis of instant noodles prepared under controlled conditions in the laboratory.

8.1 Introduction

In the earlier phases of this project it was found that a wide variety of analytical methods have been applied to the analysis of niacin in food samples. The preliminary studies described in Chapter 7 evaluated the suitability of some of the procedures for extraction and analysis of niacin in cereal-based food products. This method includes an acid/alkaline extraction and SPE followed by HPLC with UV detection and in the previous phase of the current study (Chapter 7) has been applied to wheat flour, a breakfast cereal as well as the AACC reference sample.

Currently, food fortification plays a significant role in the nutritional health and well-being of the populations of many countries (Agostini et al., 2007). On a global scale instant noodles are increasingly popular with production and consumption continuing to expand rapidly. As a result, these products can be now regarded as one of the staple foods in at least some countries and therefore they may be suitable as targets for fortification in order to ensure that dietary intakes of essential nutrients are adequate.

Based upon previous research on foods generally it is thought that niacin is relatively stable under most conditions including those involving exposure to light and heat. However, there have been no specific reports about the stability and fortification of niacin during processing of instant noodles. Recent studies concerning a range of B group vitamins and ascorbic acid have shown that these dietary components are often lost from Asian noodle products even when additional levels are incorporated through fortification (Bui & Small, 2007d, 2007e, 2008a, 2009; Hau Fung Cheung et al., 2008,

2009; Ma et al., 2007; Sanyoto et al., 2008). A further aspect of these recent studies on Asian noodles has been the demonstration that, for any one vitamin, quite different patterns of retention and loss occur for white salted, yellow alkaline and instant Asian noodles (Bui & Small, 2007d, 2007e, 2008a, 2009). Therefore, the aim of this next phase of the current study has been firstly to establish the typical levels of niacin in instant noodle samples. Further objectives have been to evaluate the stability of this vitamin during the steps in manufacture of instant noodles and to investigate the influence of factors that may cause losses of niacin during processing.

8.2 Confirmation of the validated procedure

From the previous phase of the current study (Chapter 7), the combination of an acid/alkaline extraction described by Lahély et al. (1999) combined with the SPE method of LaCroix and Wolf (2007) was evaluated in spiking studies as well as in cereal-based food products including wheat flour, AACC reference sample and a breakfast cereal. With the aim of confirming the validity of the analysis for Asian instant noodles, 1 mL of sample extract was taken after SPE procedure and spiked with 0.5 mL of 40 µg/mL nicotinic acid. Another aliquot was mixed with 0.5 mL of milliQ water. The results obtained are presented in Table 8.1. The recovery indicates that the analysis gave good recoveries of niacin.

Table 8.1 Recovery of added nicotinic acid solutions after SPE using spiking procedures

	Nicotinic acid content
Unspiked (0.5 mL of milliQ water added)	5.55 ± 0.14 µg/mL
Extract spiked with 0.5 mL of 40 µg/mL nicotinic acid	19.87 ± 0.07 µg/mL
Calculated difference between spiked and unspiked	14.32 µg/mL
Expected value based on amount of spike added	13.33 µg/mL
Recovery	107.4%

Notes 1 Results are expressed as mean ± standard deviation
2 The experiment was carried out by taking 1.0 mL of an extract of noodles and adding 0.5 mL of either water or nicotinic acid solution

In order to further validate the stage in the analysis involving SPE treatment, nicotinic acid was incorporated as a fortificant at a level of 17.53 mg/100g on a flour weight basis into the instant noodles was investigated by passing the extracts through the combination of a 500 mg C18 Sep-Pak Vac and a 500 mg SCX column used sequentially. The alternative treatment used for comparative purposes was treatment with only a 500 mg SCX column and the results are presented in Table 8.2.

Table 8.2 A comparison of the nicotinic acid content values obtained of different SPE treatments of extracts taken at selected stages of noodle processing

	Nicotinic acid (mg/100g)	
	C18 Sep-Pak Vac and SCX	**SCX**
Dough	9.04 ± 0.37	19.74 ± 0.18
Dried noodles	6.91 ± 0.86	17.01 ± 1.63

Note Results are expressed as mean ± standard deviation

The results obtained (Table 8.2) show the effectiveness of the SCX column for the purpose of clean-up whereas the combination of two SPE columns was not found to be successful in obtaining a good recovery. This can be due to some of the nicotinic acid is still bound to the C18 column. These results confirm that the use of only the SCX cartridge is sufficient to provide good clean-up of sample extracts along with good recovery of niacin added as a spike. On this basis, the procedure adopted for all subsequent analyses of noodle samples described in this thesis was that described and validated and which utilises acid/alkaline sequential extraction in conjunction with cation exchange SPE and HPLC analysis.

8.3 Laboratory studies of the influence of noodle processing on niacin contents

In the current study, instant noodles were prepared in the laboratory using approaches selected to reflect typical commercial formulations and processing practices. The detailed procedures adopted are those detailed in Chapter 6. For this phase of the study

samples were taken at each stage of processing and then the samples were analysed for moisture content, pH as well as niacin contents. The results obtained for moisture content and pH are presented in Table 8.3.

Table 8.3 Moisture contents and pH values for instant noodle samples prepared in the laboratory and analysed at different stages of processing (n=3)

Processing step	Moisture content (%)	pH
Dough	$35.0^{a} \pm 1.4$	$7.95^{a} \pm 0.06$
Sheeted dough	$31.3^{b} \pm 1.3$	$7.87^{a} \pm 0.08$
Steamed	$36.1^{a} \pm 1.4$	$7.75^{a} \pm 0.15$
Dried noodle	$9.58^{d} \pm 0.45$	$8.26^{b} \pm 0.06$

Notes 1 Results are expressed as mean ± standard deviation
2 Mean values followed by the same letter are not statistically different ($p<0.05$) within the same column

The data shows a variation in moisture contents during the processing and the primary value of these results has been in the calculation of niacin content results to a dry matter basis. These calculations were used in this phase of the current study in order to facilitate the direct comparison of results at different stages of processing. The pH values of instant noodles at each stage showed relatively little variation although there was a significant increase during the final stage of drying of the noodles. It is noted that the results obtained for pH at each stage of processing of instant noodles are quite similar to those previously reported for instant noodles (Bui and Small, 2007e). In addition, the current data confirms that there is relatively little change during preparation of the noodles. Instant noodles typically have an alkaline pH with a series of commercial products varying within the range of 6.4 and 8.2 (Bui and Small, 2007c). These contrast with white salted noodles (3.9 to 5.9) and alkaline noodles (8.3-10.3) with more kansui typically being added to the latter.

8.4 The stability of nicotinic acid in instant noodles

For the current study consideration was given to the selection of a suitable level of fortification. For this, the regulatory framework applying in Australia was chosen as this

is typically based around dietary recommendations that are, in turn established so that consumers obtain sufficient of the particular nutrient without being exposed to high levels that might entail risks to health. In Australia and New Zealand, the Standard specifically regulating the addition of vitamins and minerals to foods is Standard 1.3.2 of the Australia New Zealand Food Standards Code (Food Standards ANZ., 2010b). The maximum claim per reference quantity (proportion of the RDI) of niacin for addition to cereal flours, pasta and breakfast cereals is 25%. Therefore this level was chosen and nicotinic acid was added to the formulations at this rate. It is also noted that niacin occurs naturally in more than one molecular form. Of these, nicotinic acid was chosen as this is the form permitted for fortification purposes under the legal requirements (Food Standards ANZ., 2010b). This was added to the flour in the dry form rather than to the kansui solution. It has been confirmed from the analyses presented in Chapter 7 that the native niacin content in wheat flour is at a relatively low level. This reflects the localisation of most of niacin present in the wheat grain where it is concentrated in the aleurone and germ layers. Therefore, as has been observed for many of the vitamins and minerals in the wheat grain, niacin is removed along with the bran during milling (Ball, 2006; Gregory, 2008). The aim of the current phase of this study has been to assess the suitability of instant Asian noodles as a vehicle for niacin fortification. In order to validate the procedure and ensure the reliability of the data obtained, along with each batch of noodles prepared, the AACC reference sample was analysed in parallel.

In the initial phase, two sets of instant noodles were prepared in the laboratory under controlled conditions, and these were control samples (unfortified) as well as those fortified using nicotinic acid. The amount selected for addition to the fortified formulation was equivalent to 16.0 mg of nicotinic acid per 100 g of flour on a flour weight basis, so as to ensure compliance with the regulatory maximum value. The amount added to the initial flour as fortificant was equivalent to approximately 42.6 mg per 300 g flour corresponding to a batch of noodles. Similar chromatographic patterns were obtained for samples taken at each step in the processing of noodles and a typical set of results is shown in Figure 8.1. It is noted that for unfortified noodles, the niacin content in instant noodles represented a relatively small amount and in some chromatograms the peaks were so small as to represent less than the detection limit of this method. These latter observations confirm the results described in the previous

chapter and therefore show that unfortified instant noodles are not a significant dietary source of niacin.

In relation to the fortified noodles, the results of analysis were calculated to a dry weight basis using the moisture data presented in Section 8.3. This approach was used as it allows direct comparison of the values at each stage of processing and reflects that previously adopted in similar studies of other vitamins and their stability in Asian noodle samples (Bui & Small, 2007d, 2009). For presentation, the contents of nicotinic acid have been expressed in relative terms, as a percentage of the initial fortified level. Niacin contents for the various extracts prepared during the processing of instant noodles fortified with nicotinic acid are shown in Figure 8.2.

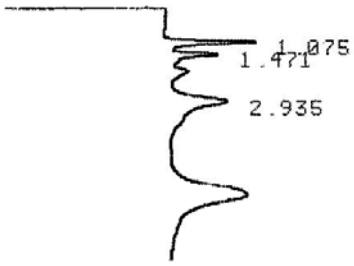


Figure 8.1 A typical chromatogram obtained for nicotinic acid in sample extracts

Notes
1. This particular chromatogram was obtained for an extract prepared from a fried noodle sample
2. The peak for nicotinic acid was identified from chromatograms obtained when solutions of the purified compound were run
3. The retention time of nicotinic acid in the chromatogram is 2.935 min

From Figure 8.2, the results show that relatively little loss occurred at each step. These results provide the first time that data has been presented in which the stability of niacin has been researched for any form of Asian noodle product. Although it appears that approximately 8% was lost during the frying step in the processing of the instant noodles, the results have been analysed statistically using one-way ANOVA and the statistical data is presented in Appendix 3. No significant difference was found (p <0.05) and so the experiments indicate that, at least under the conditions used here in the processing of instant noodles, nicotinic acid used as a fortificant is relatively stable and no significant loss occurs.

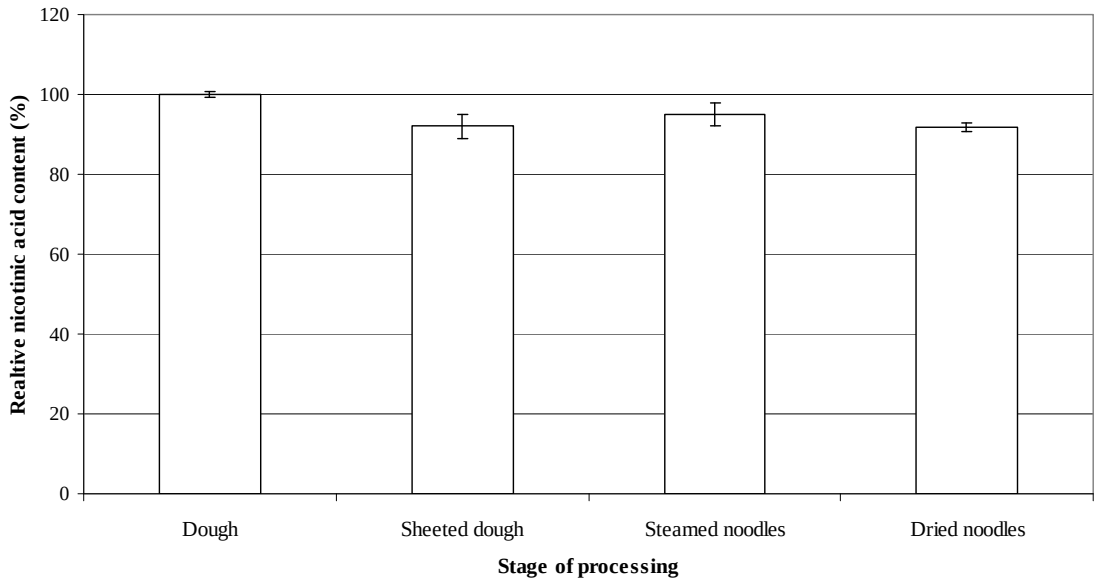


Figure 8.2 Relative nicotinic acid contents and corresponding standard deviation values at different stages during the processing of instant noodles

Notes 1 The data for the vitamin contents were first recalculated in order to express all values on a dry weight basis

2 The results plotted are relative contents with respect to that found in the dough, presented as percentage values

In considering these results it is firstly noted that, among the vitamins, niacin vitamers are generally more stable than any of the other vitamins, including both those that are considered as fat and water soluble (Gregory, 2008). In relation to the retention of vitamins in Asian noodle products, the pattern of losses for niacin contrasts with that recently found for a number of other water soluble vitamin compounds. The previous data indicates that for each of the other B group vitamins and also for ascorbic acid (Bui & Small, 2007d, 2007e, 2008a, 2009; Hau Fung Cheung et al., 2008, 2009; Ma et al., 2007; Sanyoto et al., 2008) the losses have been readily measurable. Furthermore, substantial losses occur during processing and preparation for each of the vitamins studied.

In addition to the general pattern in which niacin retention in foods is typically high, the pattern reported here for instant noodles confirms recent observations made on foods made from wheat flour. In a study of tortilla processing by Burton, Steele, Jefferies, Pike, and Dunn (2008), niacin was found to be one of the most stable of a series of vitamins and showed no significant loss during processing.

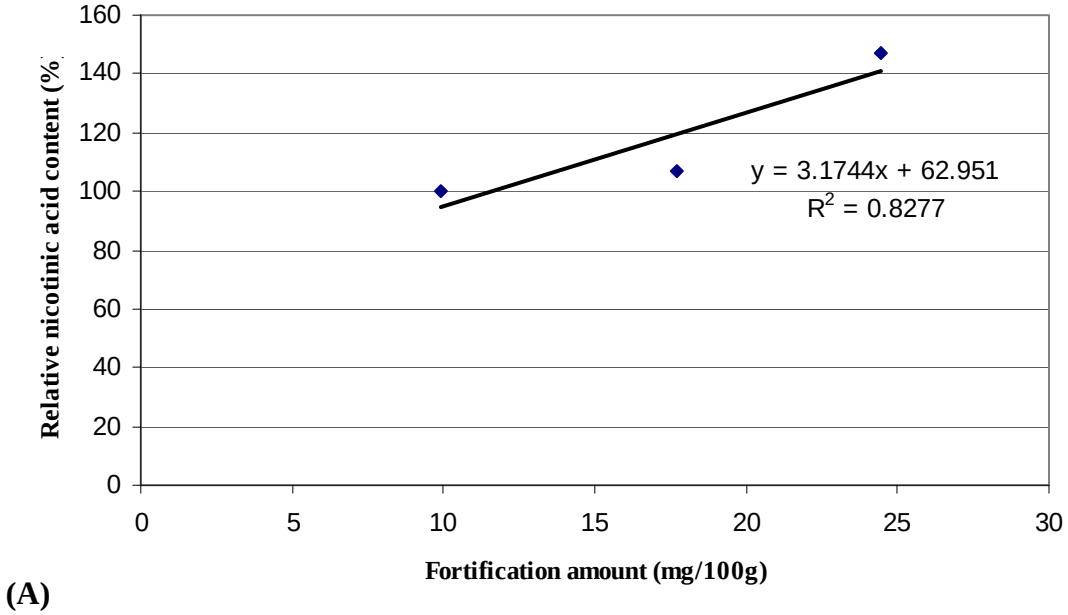
In spaghetti products, it has previously been reported that drying temperature has little influence on niacin content (Watanabe & Ciacco, 1990). In that research it was found that no significant losses were observed during drying even at higher temperatures of drying (approximately 70°C). In comparison with the deep frying of instant noodles, the temperature of drying for extruded pasta products is typically around 50°C and much longer periods of time are involved. For pasta the temperatures are much lower than used in frying of instant noodles.

From the results obtained here for the Asian noodles, it is appears that this group of products can be an effective vehicle for fortification with niacin. This can be contrasted to the previous studies on thiamin, riboflavin, folates, folic acid and vitamin B6 for which at least 40% of initial levels were lost during processing and preparation (Bui & Small, 2007d, 2007e, 2008a, 2009; Hau Fung Cheung, 2008; Hau Fung Cheung et al., 2008, 2009). In order to facilitate the fortification of foods with these vitamins, microencapsulation has been proposed and preliminary studies show that folic acid retention can be strongly enhanced using microencapsulation (Goh, Hau Fung Cheung, Al-Widyan, & Small, 2008). The technique of spray drying has been applied and the release of encapsulated components from cereal based foods is also being studied (Al-Widyan & Small, 2005; Patty, Ratanavivan, & Small, 2010). Whilst these strategies might be useful for a number of the vitamins and potentially other sensitive food components, the current study demonstrates that niacin is very stable and that no additional protective steps are required for niacin when nicotinic acid is used as the fortificant for Asian instant noodles. As a further stage of this study, the impact of fortification level on retention has also been addressed.

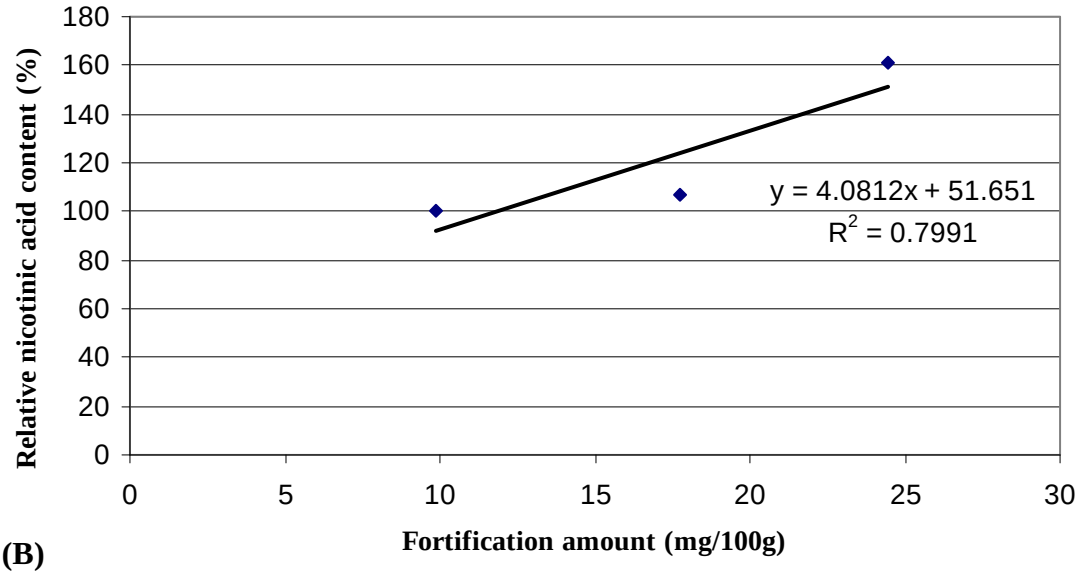
8.5 Fortification of nicotinic acid at different levels of nicotinic acid

In order to investigate the potential effect of fortification, a series of noodle samples was prepared at various levels of fortification in laboratory. Samples were taken at two stages during processing for analysis of nicotinic acid content: the dough stage (immediately after mixing) and also following deep frying. From the previous section (Section 8.4), the level of nicotinic acid chosen was 16.0 mg/100g on a dry flour weight basis which gave a readily measurable peak in the chromatographic system used here

and the result obtained showed effectiveness for fortification purposes. Therefore, in order to compare fortification rates, three levels of incorporation were selected and these were added into the formulation at rates of 9.89, 17.72 and 24.48 mg/100g on a dry weight basis with respect to the flour in the formulation. The results obtained for samples taken at the dough and deep frying stages were expressed on a dry weight basis and as relative values compared to the initial fortified level. These are presented in Figure 8.3.



(A)



(B)

Figure 8.3 Relative nicotinic acid contents and corresponding fortification amount of (A) dough and (B) dried instant noodles for different levels of fortification

The results show that when additional nicotinic acid has been incorporated into the formulation, there has been an increase in the measured amounts at both of the stages of processing. The increases seen between the lowest and the intermediate levels of addition are not as great as might have been expected, however this effect was consistently observed when the trials were repeated. The increases between the intermediate level and the highest of the three were greater. In addition the overall trend was for higher fortification levels to result in higher recoveries within the samples, as demonstrated by the regression analyses provided in Figure 8.3 where R^2 values were approximately 0.8.

The varying levels of incorporation resulted in dough and dried noodles retaining niacin corresponding to the amount that was added and there were significant differences between the levels added. Fortification with nicotinic acid has led to increases in the amount of this micronutrient found in the final product and there is a correspondingly large increase in niacin levels compared with those in unfortified instant noodles. The results clearly demonstrate that instant noodles have the potential to be fortified with niacin and thereby provide an effective dietary source and enhance intakes of this important nutrient.

8.6 Summary of results for niacin in instant noodles

In preliminary studies, the sample extraction and preparation procedures modified from those published by Lahély et al. (1999) and LaCroix and Wolf (2007) and validated in this study, gave consistent results when applied to a sample of commercial instant noodles. The approach to extraction was therefore adopted for the further study of noodle fortification. It was confirmed that the niacin level in wheat flour samples was relatively low and these are not naturally good sources of niacin. Accordingly, the stability of niacin was investigated along with the potential for fortification. In the case of the stability, no loss occurred during the processing of instant noodles. This indicates that unlike other vitamins which have been investigated recently, approaches to protecting the fortificant, such as microencapsulation, do not warrant further investigation. In addition, fortification with higher levels of nicotinic acid was effective in enhancing even further the niacin level in the instant noodles. Accordingly it is

concluded that this product has the potential to be a suitable vehicle for fortification and these foods might then be considered as good sources of niacin.

Chapter 9

Results and discussion: Preliminary assessment of procedures for extraction and quantitation of acrylamide from cereal-based foods

The purpose of this chapter is to describe and discuss the results obtained during the evaluation of procedures for analysis of acrylamide in cereal-based foods.

9.1 Introduction

Since the first reports of acrylamide formation in foods, there have been a variety of studies on methods of analysis and these have been reviewed in Chapter 3. The specific challenge in analysis has been that the levels typically found in foods are quite low. In addition, analysis and particularly extraction procedures often require modification and adaptation to particular food products. Accordingly, in the initial phase of the current study, the experiments were designed to evaluate the potential application of various approaches to the extraction and analysis of acrylamide. Selected published methods have been investigated for application to samples of instant noodles.

Among the more widely reported approaches have been HPLC and GC analyses. For this study, the methods considered have been based upon GC and various detectors including MS, FID and ECD were evaluated. This was done in conjunction with a variety of procedures for extraction of acrylamide from samples.

9.2 Evaluation of chromatographic methods for acrylamide

Initially a solution of acrylamide standard was used to investigate a series of GC procedures with the objective being to evaluate an effective and suitable procedure having sufficient sensitivity for analysis of extracts of cereal-based food samples. For this evaluation acrylamide was used as well as 2,3-DBPA as this brominated derivative has been found to assist in the quantitation of acrylamide in conjunction with particular detectors (Gertz & Klostermann, 2002; Tareke et al., 2002; Zhang et al., 2006).

9.2.1 Evaluation of GC-MS methods for acrylamide

GC-MS was firstly evaluated using a published procedure reported by Zhang et al. (2006). This was based upon use of a BPx5 column (length 30 m × ID 0.25 mm × film thickness 0.25 μm) and a temperature gradient which is presented in Table 9.1. For the trials, a series of standard solutions of acrylamide were prepared within the range of 10-100 μg/mL and further details of the instrumentation and conditions used are provided in Section 6.9. The results obtained from the chromatograph did not show a significant peak, even when concentrations of 100 μg/mL were run. On this basis, alternative approaches were considered.

Table 9.1 Oven program used for the analysis of acrylamide using a GC-MS procedure

Program step	°C/min	°C	Time (min)
Initial		40	3
Ramp	20	40-250	10.5
Post Run		220	3.50

9.2.2 Evaluation of SPME methods for acrylamide

The second approach considered was adopted from Lee et al. (2007). This involved direct immersion SPME, used as a clean-up technique following by GC coupled to FID. For this, samples of food materials were prepared by extraction with 100 mL of water at room temperature using 10 g samples, with supersonic nebuliser for 30 min followed by centrifugation at 5000 rpm for 10 min. Aliquots (1.5 mL) were diluted with 15 mL of water and mixed with 15 mL of a buffer solution (adjusted to pH 7.0). Then an SPME fiber was directly immersed into the resultant solution. For the selection of the fibre it is noted that a number of options are available commercially. Of the several types of SPME fibres currently available, the choice is based upon principle that “like dissolves like”. Hence a Carbowax/divinylbenzene (CW/DVB, 65 μm) fibre from Supelco Company was selected as having a polar coating, the polarity of the fibre material is high, matching that of acrylamide.

The CW/DVB fibres were used following a conditioning step which was carried out according to the recommended procedure of the manufacturer. This preparative step was considered to be important so that any contaminants which might give a high background in the chromatogram can be removed (Penton, 1999). Cleaning was done by inserting the fiber in an auxiliary injection port of the GC instrument with a temperature of 220°C for a period of 10 min. Then the fibres were used for absorption of acrylamide by dipping into the solution, with an extraction time of 20 min at room temperature.

For the analysis, the SPME procedure was applied to extracts of samples and also to a series of standard solutions. The SPME fibres were then analysed directly for acrylamide contents on a Shimadzu GC-17A system, with FID detector. The instrument was fitted with a Solgelwax column (30 m length × ID 0.25 mm × thickness 0.25 μm) and the carrier gas was Hydrogen flowing at a rate of 1.5 mL per min. The injector was operated in the spiltless mode for one min with an injector temperature of 220°C. The oven temperature was set at 50°C and held for one min followed by a linear temperature gradient of 5°C per min up to 220°C. An example of the calibration curve obtained is presented in Figure 9.1 and typically excellent linearity was achieved with a correlation coefficients of $R^2 \geq 0.99$.

However, a further step was to apply this procedure to extracts of cereal-based foods. Despite a series of attempts, this was not found to be practical for such samples, regardless of whether the sample preparation was carried out with or without spiking which involved addition of 1 mL of 1000 μg/mL acrylamide at the beginning of sample preparation. Samples of both potato chips and cornflakes were also investigated with a higher level of spike (corresponding to 5 mL of 1000 μg/mL acrylamide). In all cases, the consistent observation was that during immersion of the SPME fiber, the material rapidly became swollen and as a result breakage occured. Although various attempts were made to utilise the potential advantages of the SPME fibres, these were not successful for the samples trialled. Hence, alternative approaches were considered next.

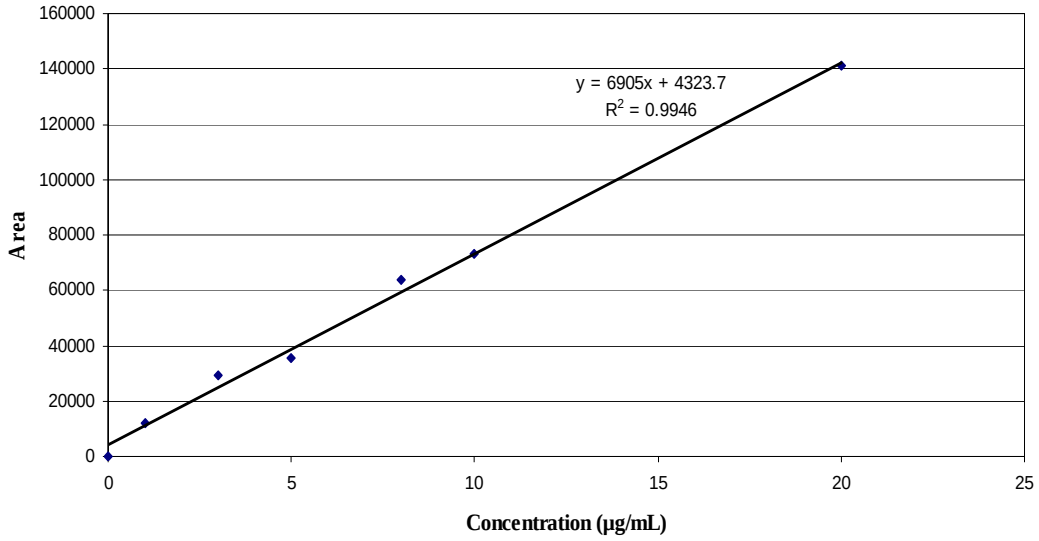


Figure 9.1 Calibration curve of acrylamide on GC-FID using SPME procedure

9.2.3 Evaluation of GC-NPD for analysis of acrylamide

In a further preliminary evaluation of GC analysis methods for acrylamide, the use of a GC coupled to an NPD was applied to standard solutions of 2,3-DBPA. The principle of NPD is similar to that of FID with a rubidium or caesium silicate bead situated close from the hydrogen flame, in a heater coil. This arrangement has the effect of adding alkali metal vapour into the flame so that the detector responds to compounds containing either phosphorus or bonds between carbon and nitrogen atoms (Flanagan et al. 2008). This approach has potential for application in acrylamide analysis as 2,3-DBPA contains C-N bonds and Yasuhara, Tanaka, Hengel, and Shibamoto (2003) indicated that GC-NPD provided satisfactory results when compared to LC-MS. In addition, EL-Ghorab (2006) used GC-NPD for determine acrylamide in conjuction with SPME and the system was found to provide excellent chromatograms for selected food samples. An example of a typical chromatogram is shown in Figure 9.2 and this demonstrates a relatively small peak even at the concentration of 20 µg/mL. From these consistent observations, it was concluded that the system described here with the NPD was not suitable for the levels of acrylamide in the noodle samples being tested.

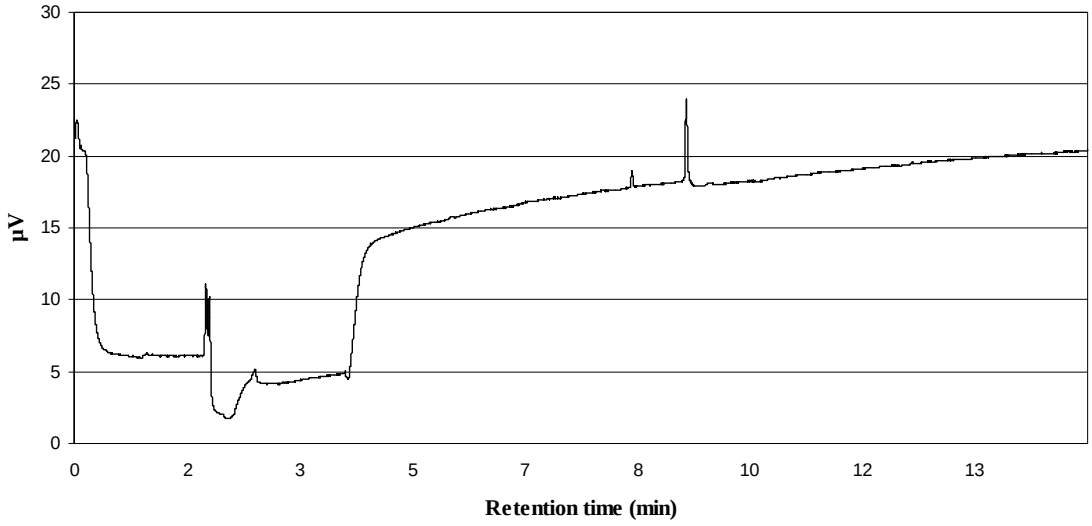


Figure 9.2 Chromatogram of a standard solution of 2,3-DBPA with NPD

Note This compound is a brominated derivative of acrylamide and was prepared at a concentration of 20 µg/mL with the retention of 9.06 min

9.2.4 Evaluation of GC-ECD for analysis of acrylamide

A further approach to analysis of acrylamide was the method of Zhu et al. (2008) and this involves sample preparation by derivatisation with hydrobromic acid and saturated bromine-water followed by liquid-liquid extraction using ethyl acetate. The procedures are described in detail in Chapter 6. The analytical procedure was firstly applied by running the pure chemical 2,3-DBPA in the form of a standard. A typical chromatogram obtained for this compound is presented in Figure 9.3. It is noted that the retention time of 2,3-DBPA was approximately 8.87 min and this showed excellent chromatographic separation from all other components present. Although other minor peaks were consistently observed in the chromatograms, when varying concentrations of the compound were analysed, it was only this peak which increased linearly in area. An example of a calibration curve obtained using solutions having a range of concentrations of 2,3-DBPA is shown in Figure 9.4. The results typically showed a high degree of linearity (correlation coefficient $R^2 \geq 0.99$).

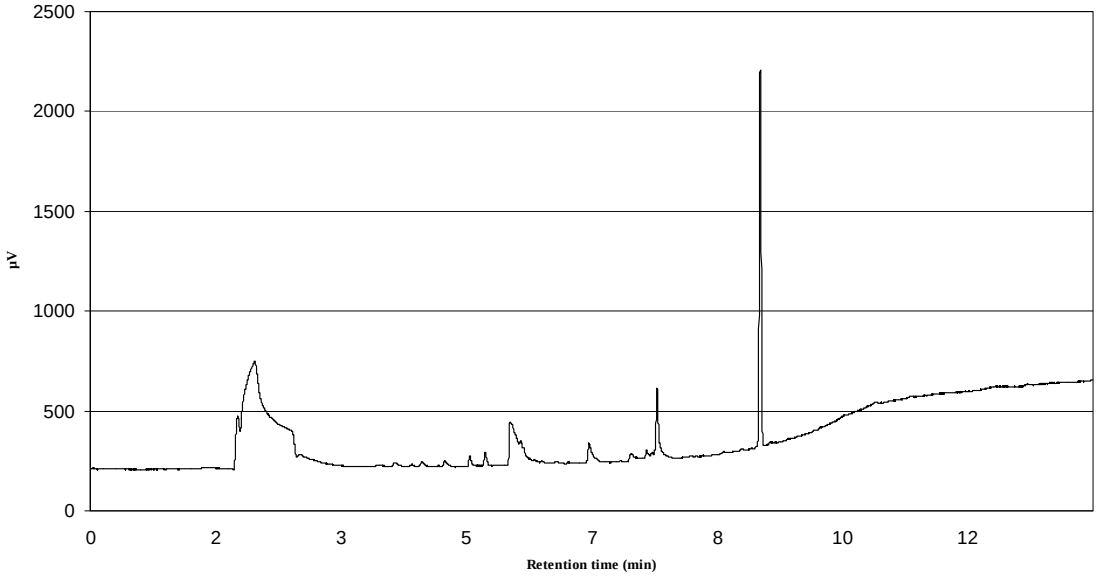


Figure 9.3 Chromatogram of a standard solution of 2,3-DBPA with ECD

Note This compound is a brominated derivative of acrylamide and was prepared at a concentration of 0.05 µg/mL eluting with the retention time of 8.87 min

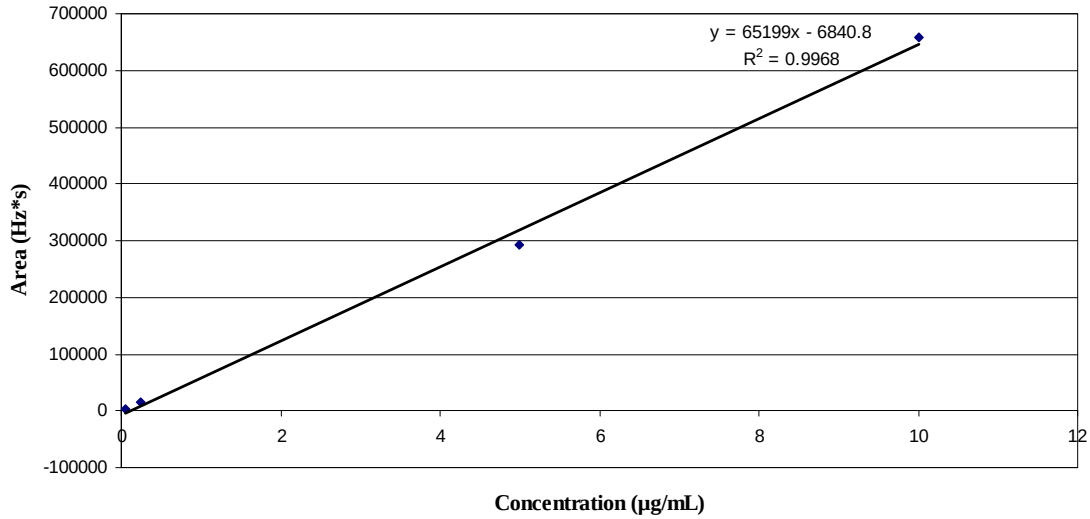


Figure 9.4 A calibration curve for 2,3-DBPA standard solution

In the original report (Zhu et al., 2008), the food samples analysed included fried instant noodles, non-fried instant noodles, potato chips, and biscuits. In that study, it was found that the standard addition method was required in order to effectively quantitate the acrylamide content of the samples being analysed. It was suggested that the technique of

standard addition could be used to identify the target analyte because only the corresponding target peak increased with the addition of the standard.

In the current study the approach using standard addition was trialled and was found to provide reasonable quantitation of acrylamide in instant noodle samples. However, the results obtained often showed variability in areas for the peak of 2,3-DBPA, particularly when replicate injections of a sample were compared. Accordingly it was decided to investigate whether the use of an internal standard would provide an advantage for the analysis. In selecting chemicals which had potential for this purpose, it was necessary to consider halogenated compounds on the basis that the detector being used was on ECD.

In seeking a suitable internal standard it is important that the compound is not a component of any sample to be analysed and also that it does not react with any other molecules in sample extracts. Moreover, it should elute near the peak of interest but not overlap any sample peaks (McNair & Miller, 2009). For the current study, 1-chloronapthalene was chosen for evaluation.

When 1-chloronapthalene was used along with the standard addition method and applied to a sample of instant noodles, effective chromatographic separation was achieved and a typical example of a chromatogram is presented in Figure 9.5. The internal standard was found to provide particularly symmetrical peak shape and a typical retention time of 8.69 min when used at a concentration of 5 µg/mL this facilitated effective quantitation of the halogenated derivative of acrylamide. The corresponding calibration curve was prepared based upon the ratio of internal standard to the acrylamide derivative 2,3-DBPA (Figure 9.6). The typical results consistently showed excellent linearity (correlation coefficients, $R^2 \geq 0.99$).

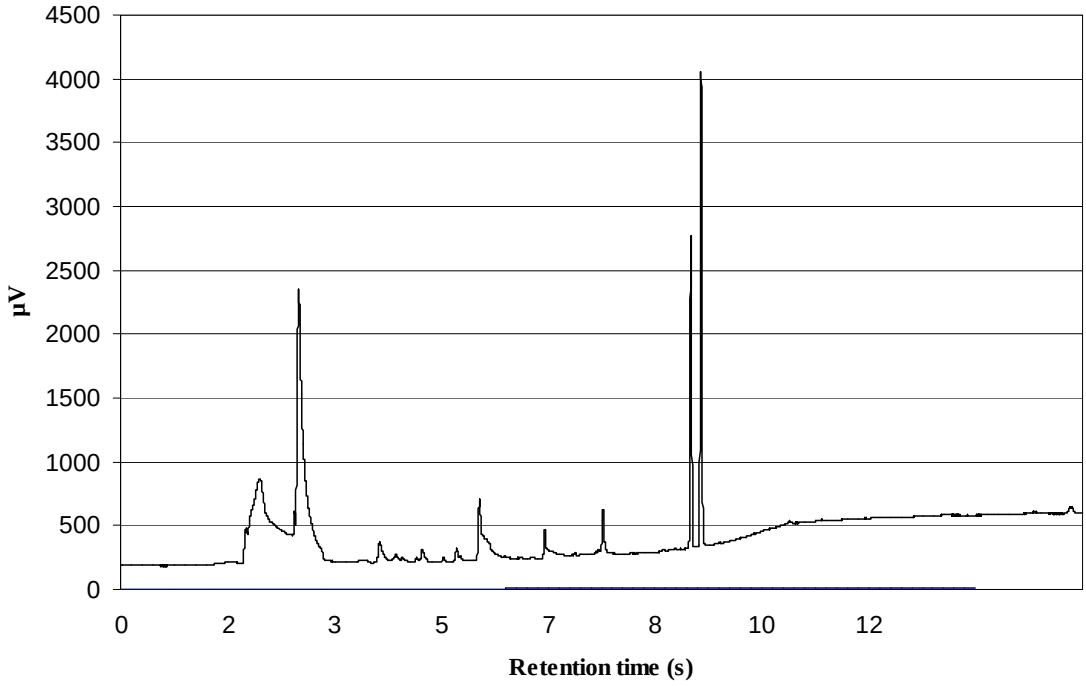


Figure 9.5 Chromatogram obtained using ECD for noodles prepared by the standard addition method with 1-chloronapthalene as internal standard

Notes 1 The chromatogram shown is for standard addition at a level of 0.1 µg/mL of acrylamide working solution
2 2,3-DBPA has a retention time of 8.86 min
3 The internal standard, 1-chloronapthalene, was used at a rate of 5 µg/mL and the corresponding retention time is 8.69 min

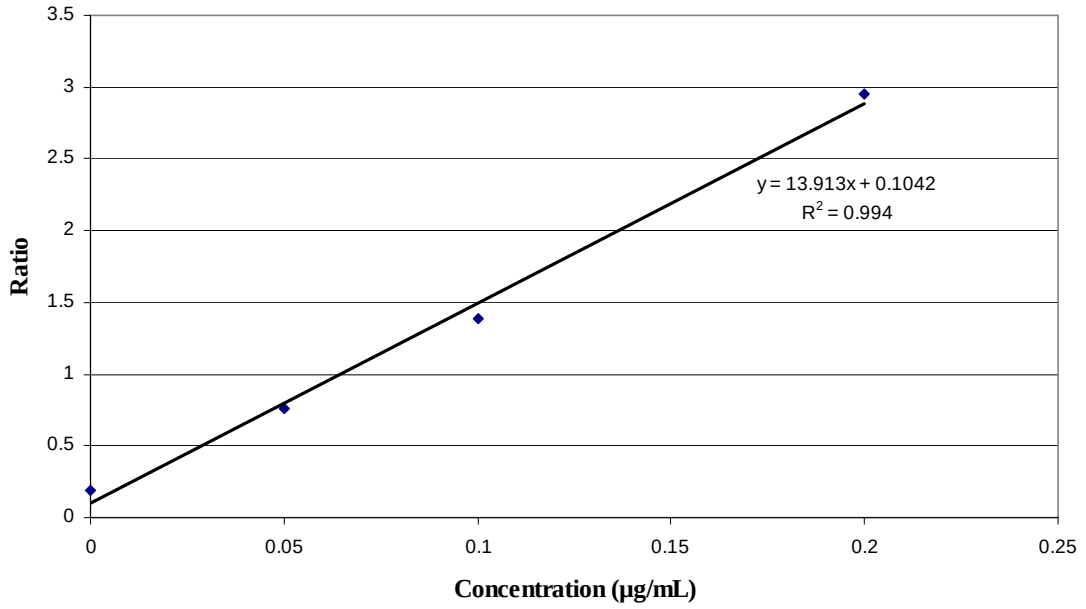


Figure 9.6 An example of a calibration curve prepared using the standard addition method for a sample of instant noodles

Hence in the current study, standard addition in combination with spiking of sample matrices was utilised for the purpose of measuring for the brominated acrylamide derivative. This approach was specifically applied to selected food samples and for this purpose potato chips and peanut butter were chosen because they are expected to contain acrylamide (Chen et al., 2008; Ölmez et al., 2008). Potato chips were purchased from a supermarket in Melbourne and peanut butter was supplied by NIST and further details are presented in Chapter 6 (Table 6.4).

For the analysis of the samples, acrylamide contents in potato chips and peanut butter were found to be 446 and 263 μg/kg, respectively. These levels of acrylamide were higher than the expected values (59 and 87 ± 7.8 μg/kg for potato chips and peanut butter respectively) and therefore, a further evaluation was carried out by measuring the recovery of acrylamide with a spiking study.

9.3 The repeatability of analytical results obtained in the analysis of samples and reference materials

For this evaluation, acrylamide was added to samples at the commencement of the extraction procedure. A series of batches of samples were used and addition was at a level close to that of the known acrylamide concentration of each of the food samples used. For the peanut butter, 1 mL of 0.8 μg/mL acrylamide was added while 1 mL of 0.5 μg/mL acrylamide was spiked into each flask for the instant noodles. The results obtained for unspiked and spiked sub-samples were analysed and these are presented in Table 9.2. This also includes values calculated to allow for the amount of acrylamide in samples and the results demonstrate that good recoveries were obtained from both of the samples used for this evaluation (Table 9.2). In contrast, the previous section on analysis of NIST peanut butter sample is 3 times higher than expected value. This probably due to the protein and fat contents was very high. Thus these components may effect on acrylamide content.

In summary, from the thorough consideration and evaluation of alternative analytical procedures, many different approaches were reviewed and a number evaluated. Some of the GC-based methods were found to be insufficiently sensitive or in some other way to be unsuited to the extraction and analysis of acrylamide in Asian instant noodle

samples. Subsequently, a procedure was adapted, enhanced and validated which was found to allow for the analysis of instant noodles. Whilst reasonably repeatable values have been obtained, one aspect of the overall procedure which is not immediately apparent, but warrants mention is the limitation on throughput of samples. The necessity of using the standard addition method to overcome matrix interference, along with the application of derivatisation and the partitioning of brominated compounds into a non polar solvent, effectively result in the maximum throughput of one noodle sample in a three-day period. In this context it is also noted that, in this time period, triplicate analyses were carried out, and this was the minimum level of replication used for all of the samples for which data is reported in further stages of this study.

Table 9.2 Recovery of acrylamide following spiking of two food samples

	Peanut butter	**Instant noodles**
Mean acrylamide content (before spiking, μg/kg)	237	53.7
RSD (%)	15.6	14.1
Spike level (μg/kg)	80	50
Mean acrylamide content (after spiking, μg/kg)	327	97.7
RSD (%)	13.4	14.0
Recovery (%)	118.4	88.0

Note Samples used for this experiment were NIST reference sample (peanut butter) and instant noodles (Mama brand)

9.4 Investigation acrylamide content in commercial instant noodles

The investigation was continued with a series of analyses of commercial noodle samples using derivatisation following by GC-ECD. Commercial noodles analysed in this study were purchased from supermarkets in Melbourne and the basis of selection was to include samples representing as wide a range of countries of origin as possible. Details of the noodle samples are presented in Chapter 6 (Table 6.8). The results of acrylamide

content and the pH values of the different brands of instant noodles are presented in Table 9.3.

Table 9.3 Comparison of acrylamide content with different brands of instant noodles

Brand	Acrylamide content (µg/kg)	RSD (%)	pH
Mama	59.6 ±8.4	14.1	7.1
Maggi	11.6 ± 6.3	54.8	6.9
Mr. Kon noodles	37.3 ± 3.0	7.9	7.2
Doll instant noodle	77.4 ± 3.8	4.9	7.5
Nissin	54.8 ± 6.1	11.1	7.7
Megah Mee EE FU noodles	19.4 ± 1.2	5.9	9.1
Megah Mee Claypot Ee Mee	48.9 ± 8.2	16.9	9.1
Nong shim	37.2 ± 8.2	22.0	7.3

Note Acrylamide content are expressed as mean ± standard deviation

A wide range of acrylamide contents was found in the commercial instant noodles ranging from 11.6 to 77.4 µg/kg. The acrylamide levels in instant noodles are not high compared to those of some other food products particularly those made from potatoes (compare with Table 3.1), in which acrylamide contents as high as 5200 µg/kg have been reported. Despite this, for a consumer who eats instant noodles on a daily basis, these products might also contribute substantially to the total acrylamide intake.

In relation to the pH values (Table 9.3) there appears to be no significant correlation with acrylamide content in the commercial products. This shows that pH is not the primary factor which might be used to explain the differences in the acrylamide contents of the various brands of commercial instant noodles.

Based upon the available evidence reviewed in Chapter 3, and relating to the factors believed to influence Maillard reactions generally, along with the relatively recent studies specifically investigating acrylamide formation, a number of factors may be relevant to the results presented in Table 9.3. In addition to pH, it is expected that the combined impact of factors including frying time and temperature, raw materials, formulation as well as product composition may be important. These issues are considered in the following chapter.

9.5 Summary of the investigation of procedures for acrylamide analysis

A series of evaluations has been carried out in order to establish an approach that can reliably be applied to Asian instant noodles. Various GC procedures reported in the literature were trialled and these encompassed GC coupled with different detectors (MS, NPD, FID and ECD). A number of the methods considered appeared to lack the sensitivity required for the current purpose and these included the systems based upon GC-MS, GC-NPD and GC-FID which could not be readily validated to apply to cereal food products. In addition, for sample extraction and preparation SPME followed by GC-FID was found to be unsuitable for cereal-based food products due to swelling and breakage of the fibres.

Ultimately it was found that derivatisation involving bromination in conjunction with GC-ECD was effective and useful for analysis of acrylamide, giving excellent peak shape and clear chromatographic separation. The procedure for sample extraction was studied and it was found that the standard addition approach was required in order to minimise matrix interference effects. It was proposed that the use of 1-chloronaphthalene would provide a further enhancement and this was shown to facilitate quantitation of acrylamide contents. Good recoveries were obtained when the methods for extraction, derivatisation and analysis were applied to selected food products. For validation, a standard reference sample was used as well as a sample of commercial instant noodles. Whilst relatively repeatable data can be achieved, the method adapted does have significant limitations in terms of sample throughput.

The validated procedure was applied to a range of instant noodle samples and the acrylamide contents were found to vary quite widely. The contents of at least some

brands were sufficiently high that they might contribute significantly to acrylamide intakes of consumers who regularly eat these products. The next phase of the current study has been designed to investigate the factors that might influence acrylamide formation so that strategies for minimisation might be developed for the processing of Asian instant noodles.

Chapter 10

Results and discussion: The impact of processing conditions on acrylamide formation in instant noodles

The purpose of this phase of the research project has been to investigate the impact of processing including frying temperature and time, pH as well as water activity on acrylamide formation. In addition, the impact of asparaginase on acrylamide formation in instant noodles has been evaluated.

10.1 Instant noodles made on a laboratory scale

The procedure of making instant noodles adopted for this phase of the study is the same as that adopted and described in the earlier phase where niacin retention and fortfication. The details are described in Section 6.3 and for each batch of noodles a flour weight of 250 g was used as this was sufficient to allow for triplicate analyses of acrylamide content for each control and treatment sample. For the instant noodles made in this phase, the impact of frying time and temperature were investigated by measuring acrylamide content, pH, product colour as well as water activity.

10.2 Introduction to investigate the impact of processing on acrylamide content

Since the initial discovery of acrylamide in foods, a variety of factors has been found to influence formation of the toxin and these have been reviewed in Chapter 3 (Section 3.9). In setting up the laboratory methods for noodle preparation for this phase of the study one particular challenge encountered was associated with maintaining the temperature of oil during the deep frying stage of processing. The pieces of equipment used in the laboratory for noodle preparation are shown in Chapter 6 (Figure 6.1) and initially, a small deep fryer designed for domestic use (Figure 6.1E) was trialled and found to be unsatisfactory. It was found that upon addition of the noodle sample to the oil, the temperature typically dropped by as much as 15°C and it was not possible to control this by reducing the size of the noodle sample being processed. This reflected the fact that for each replicate of a particular treatment, a sample size of 150 g was

required to allow for triplicate extractions of acrylamide as well as providing sufficient sample for the other analyses carried out.

A larger deep fryer was then trialled and in this an increased volume of oil was used and the heating capacity was also greater. The way in which this unit (Figure 6.1F) was utilised for the initial experiment was that a constant frying temperature was chosen of 145 ± 5°C and the frying time was varied between 10 and 300 s. For all experiments, the temperature was carefully monitored and recorded during each frying period and reported in terms of the average of the actual temperatures measured. Then, the samples were analysed for acrylamide content, water activity, moisture content, pH as well as colour characteristics.

Moisture content was determined following the AACC method and the results were calculated from the weight difference from before and after drying of samples. The water activity meter utilises the dew point principle and gives a surface equilibrium relative humidity of the products. The data on moisture content and water activity were obtained from triplicate analyse. The results obtained for water activity and moisture content are presented in Tables 10.1 and 10.2.

Table 10.1 Water activities and moisture contents of deep fried noodles processed at different frying times with temperature held constant at 145°C

Frying time (s)	Moisture content (%)	Water activity
10	14.47^{a} ± 0.54	0.5180^{a} ± 0.00
45	3.92^{b} ± 0.05	0.5040^{a} ± 0.00
120	2.27^{c} ± 0.10	0.4580^{b} ± 0.03
200	2.84^{c} ± 0.10	0.2740^{c} ± 0.03
300	3.15^{bc} ± 0.29	0.2606^{c} ± 0.01

Notes 1 Results are expressed as mean ± standard deviation
2 Mean values followed by the same letter within the same column are not statistically different ($p<0.05$)

Table 10.2 Water activities and moisture contents of deep fried noodles processed at different frying temperatures with time held constant at 45 s

Frying temperature (°C)	Moisture content (%)	Water activity
128.3	$7.40^{a} \pm 0.16$	$0.4890^{a} \pm 0.00$
137.0	$6.49^{b} \pm 0.47$	$0.4640^{b} \pm 0.00$
145.7	$3.92^{c} \pm 0.05$	$0.5040^{c} \pm 0.01$
159.7	$5.29^{d} \pm 0.24$	$0.4280^{d} \pm 0.01$
174.0	$3.09^{e} \pm 0.08$	$0.4510^{e} \pm 0.01$

Notes 1 Results are expressed as mean ± standard deviation
2 Mean values followed by the same letter are not statistically different ($p<0.05$) within the same column

The frying step at 145°C for periods of between 10-300 s resulted in a decrease in the moisture content from 14.5 to 3.2%. On the other hand, when the frying stage was carried out for a constant frying time of 45 s increasing the temperature from 128 to 174°C caused a corresponding drop in the moisture from 7.4 to 3.1%. The variation found in the current study confirms that differences in the time and temperature of deep frying directly affect the moisture content of the final product. This reflects the fact that deep-frying is a dehydration process that rapidly and effectively evaporates water from the products. In contrast, at a constant frying time of 45 s, even with increasing frying temperature, the water activity remains relatively constant. This may be due to times of 45 s being insufficient to reach stage three of frying where stage three refers to an increasing in the temperature up to the frying oil temperature which is requires an extended frying time. Further investigation of the impact of frying on acrylamide was carried out during the following experimental phases.

10.3 The influence of frying time on acrylamide content

A number of factors identified as being likely to influence acrylamide contents were then evaluated and the primary factor relates to frying conditions. For the current investigation, frying time was firstly examined using the same analytical procedures for

acrylamide that were developed and described in the previous chapter for the evaluation of commercial instant noodles. Frying time was varied between 10 and 300 s with a constant temperature of 145 ± 5°C and the results of acrylamide analysis are presented in Figure 10.1.

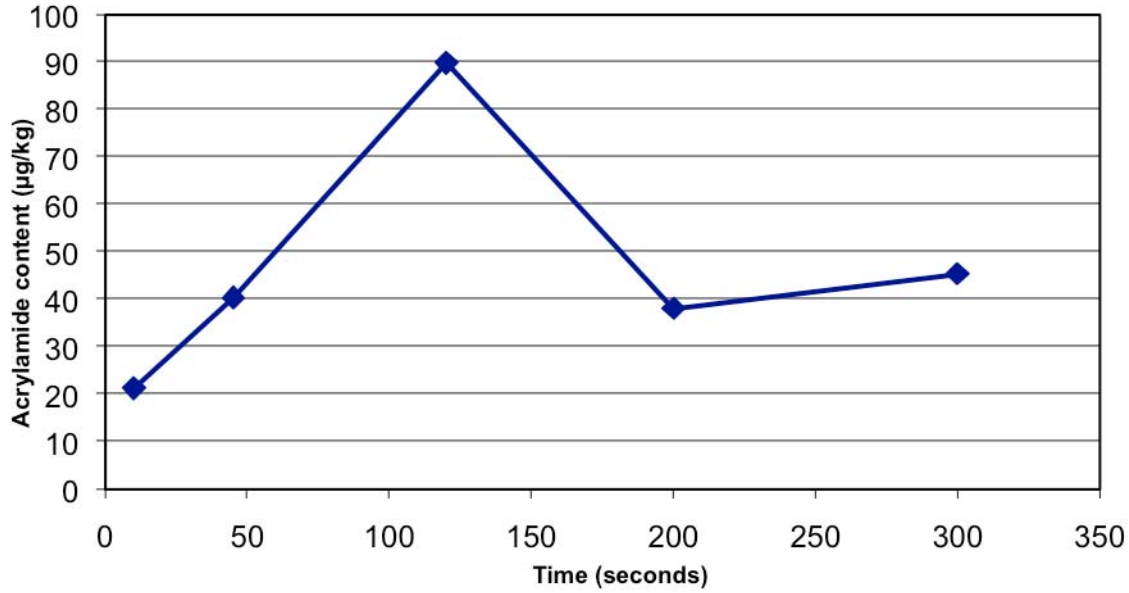


Figure 10.1 Acrylamide content of instant noodles at different frying times with temperature held constant at 145 ± 5°C

These results show that very soon after the commencement of frying at 145°C, acrylamide had formed and the amount found was 21.3 µg/kg. With continued frying, the content reached an apparent maximum of 89.9 µg/kg for a frying time of 120 s and this was then followed by a decrease to 38.1 and 45.4 µg/kg for 200 and 300 s, respectively. These results indicate that acrylamide content decreased during extended frying.

It is likely that the pattern shown in Figure 10.1 is effectively the result of at least two processes occurring simultaneously. Firstly, acrylamide is formed from the precursors present in the formulation and as the temperature rapidly increases within the noodle strands, the production of acrylamide would be expected to increase reflecting the typical influence of temperature upon the rate of any chemical reaction. A second occurrence will be the evaporation of acrylamide as the temperature within the noodle

approaches that of the oil within the deep fryer. As acrylamide has a boiling point of 192.6°C (Budavari, 2001) the acrylamide content is expected to decline and the data demonstrates that the loss is relatively rapid once the time of frying exceeds 200 s.

For the purpose of comparison, there are no other published data in the scientific literature on fried noodle products. However there have been various studies of potato chips. The results obtained in this current study with instant noodles are in accordance with those of previous studies by Gökmen and Palazoğlu (2009) and Rydberg et al. (2003), in which prolonged heating of potato strips at higher temperature resulted in lower acrylamide contents and Bråthen and Knutsen (2005) found a similar trend in a cereal model system. It was suggested that observations of declining acrylamide contents probably reflected either degradation or polymerisation of the acrylamide following its formation in the early stages of frying. In addition it was suggested that acrylamide reacts further or is eliminated through evaporation, or possibly both of these are occurring (Rydberg et al., 2003).

In considering the chemical changes that occur during the frying of foods, there are a number of other issues to be considered. Firstly it is noted that the formation of acrylamide occurs very quickly in the instant noodles: even after ten seconds of frying, readily measurable levels of the toxin have formed (Figure 10.1). In the deep frying process, the characteristics of the food samples are also important. The dimensions of the potato samples reported previously are totally different from those of instant noodles and the relevant aspects might include the surface area as well as the distance from the center to the surface. This may explain the relatively long times of frying that elapse before the levels of acrylamide reach a peak during the frying of potato chips (Romani, Bacchiocca, Rocculi, & Dalla Rosa, 2009). Acrylamide levels continued to increase for frying times of up to sixteen minutes, although the relative quantities of chips and oil were also found to be important.

The studies reported by Taubert, Harlfinger, Henkes, Berkels, and Schomig (2004) showed that acrylamide formation in potato products of various shapes was highest where there was a high surface to volume ratio. In addition, there were incremental increases in acrylamide with increasing temperature of frying. On the other hand, high temperatures during frying associated with prolonged frying time for the potato pieces

with low surface to volume ratio ultimately resulted in a decrease in the amounts of acrylamide remaining in the final product.

A further factor is the impact of moisture content and water activity. In the time since acrylamide was first reported in food it has been reported that the most common mechanism of formation is that of a Maillard type reaction. Therefore earlier studies on the relationship between moisture in foods as this impact upon to the rate of browning reactions may be relevant to the current observations on acrylamide formation.

It was previously shown that the maximum browning rate typically occurs at water activity values between 0.3 and 0.7 and that the rate does also depend on the specific properties of the food product. The water activity of the various samples of instant noodles all fall within the range originally reported as corresponding to maximum rates for Maillard reactions, regardless of the frying time (Table 10.1) and temperature (Table 10.2). Eichner and Karel (1972) originally proposed that the loss of lysine is a Maillard type reaction and so is related to the rate of browning reaction. The effect is one of increased rates with increasing water activities except in the system having low water contents (Figure 10.2). It is thought that the decline in rate reflects the restricted mobility of reactants which is significantly impeded at low water activities (Eichner, 1975; Eichner & Karel, 1972).

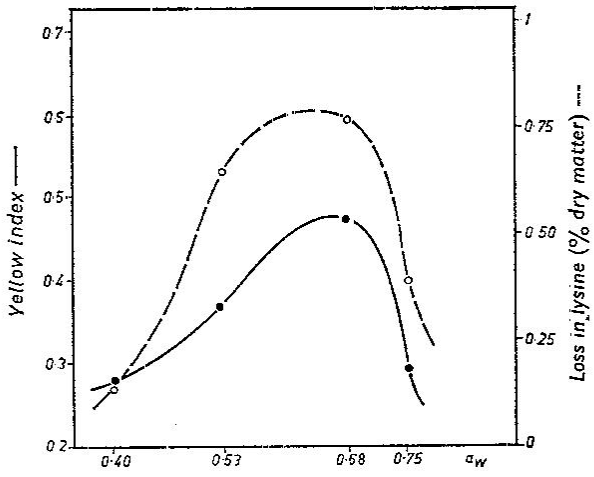


Figure 10.2 Loss in lysine and brown colour development in dried milk as influenced by water activity
Note Source of information was Eichner (1975)

The formation of acrylamide also depends upon the moisture content. In some food systems including a potato model, acrylamide formation appears to be more strongly related to the moisture content rather than the water activity (Mestdagh et al., 2006). The moisture content also had a strong affect on the activation energy of browning as well as acrylamide formation and decreasing moisture contents led to increasing acrylamide formation (Amrein et al., 2006). In a low moisture system, the results of Elmore, Koutsidis, Dodson, Mottram, and Wedzicha (2005) indicated that acrylamide formation was inversely proportional to moisture content in the cases of moisture content below 5% in cakes made from potato, rye and wheat flour. Significant increases in acrylamide formation were observed following processing by prolonged heating. Whilst this appears to contrast with the results obtained in the current study, it is noted that published data in the recent literature indicate that a number of factors may be important.

For example, Robert et al. (2005) found that the water activity does not seem to be a significant parameter for acrylamide formation in a model system based upon glucose and asparagine. Low moisture samples were prepared at different water activities by freeze drying and equilibration with selected salts. The results indicated that the physical state of the material, the temperature and particularly the reaction time were more important determinants of acrylamide formation than water activity in the amorphous system.

In summary of the results on frying time from the current study, acrylamide content increased as frying was continued up to a maximum value which was observed at approximately 120 s. After this point the formation decreased and the content declined sharply with extended frying. This probably reflects one of a number of effects including a depletion of one or more of the precursor compounds, the chemical degradation of acrylamide which is a relatively reactive compound or the evaporation of acrylamide at the elevated temperature, particularly as the central core of the noodle strands reached the temperature of the oil in the deep fryer. It is quite likely that a combination of these factors is important and the rapid change in moisture content during the deep frying process, rather than water activity, are probably also contributing to the observed effects. In relation to the practical implications of these results, it

appears that prolonged frying time can be one of the strategies to control or minimise acrylamide content of instant noodles. However, there are a number of factors that influence the formation including the frying temperature and this was further investigated in the next phase of the study.

10.4 The influence of frying temperature on acrylamide content

The frying temperature was also identified as a key factor for acrylamide formation as reviewed in Chapter 3 (Section 14). A series of noodle samples were prepared by deep frying at constant time of 45 s which was selected as a frying time commonly used both in laboratory and commercial contexts (Moss, Gore, & Murray, 1987). As has been described earlier (Section 10.2) the actual frying temperature values were measured at the commencement and completion of the frying period and the average values used in the presentation of the results. The temperatures were varied between 127 and 175°C and the results obtained are presented in Figure 10.3.

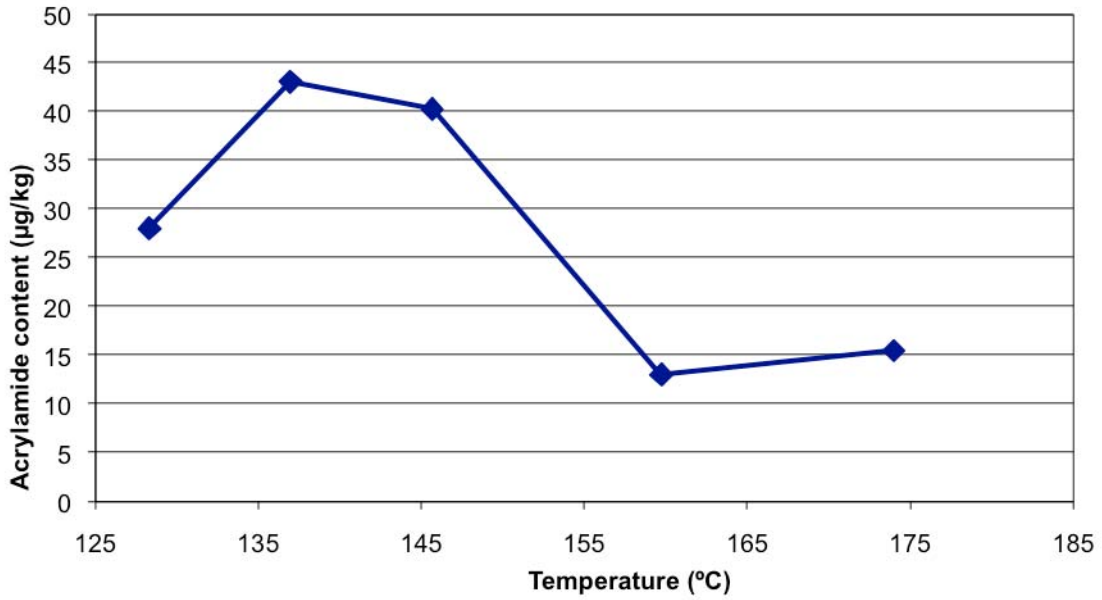


Figure 10.3 Acrylamide content of instant noodles at different frying temperature with time held constant at 45 s

These results show that the acrylamide content initially increased at hotter temperatures and reached a maximum in the range of approximately 137-145°C. With further

increases, the acrylamide then reduced to 13.1 and 15.5 μg/kg at temperatures of 159.7 and 174.0°C, respectively.

In assessing these results, acrylamide has been formed at each of the temperatures studied. Even at the lowest temperature acrylamide was readily measurable and this is consistent with the expectation that the rate of Maillard reactions will be very low at 100°C but will increase rapidly as temperature increases. The higher levels of the toxin measured at temperatures of around 135 to 145°C reflect the typical impact of temperature on the rate of chemical reactions. However, as higher temperatures were employed, moisture content dropped and the higher temperatures caused higher rates of evaporation of the acrylamide that had formed.

In relation to recent studies of various other food products, Amrein et al. (2006) found that acrylamide formation in low moisture systems including potato chips was strongly dependent upon temperature. In one of the earliest studies of acrylamide formation in foods, a dilute aqueous environment was evaluated and the effect of temperature was observed. In this system, acrylamide was not formed from asparagine at 100°C, but as long as the temperature reached 120°C, formation was found to occur. Only at relatively high temperatures (approx 165-170°C) was the apparent production of acrylamide seen to decrease in this glucose and asparagine model system (Mottram et al., 2002). In accordance with these previous studies, Becalski et al. (2004) found that the acrylamide content in dry systems was reduced when the temperature was increased from 155 to 185°C. In addition, a decreased acrylamide content in fried potatoes was reported during processing at high temperature with long times for samples of potatoes having a high surface area (Taubert et al., 2004).

In contrast to the results obtained here for instant noodles and the literature reports described above, a number of other studies have found that an increase in frying temperature leads to increases in acrylamide content. These observations relate especially to French fries (Grob et al., 2003; Rydberg et al., 2003) potato crisps processed in a hot oven as well as hamburgers (Tareke et al., 2002). Gökmen et al. (2006) found that both increasing time and temperature of frying led to increased acrylamide contents as an exponential function in French fries. These observations were

explained as probably being due to heat transfer from the heating medium to the surface of products during frying.

In comparing the results for instant noodles and those previously reported for French fries, the former have a high relative surface area. In addition, moisture is transferred from the interior to the surface as a result of high temperature and low moisture content especially as the surface rapidly becomes drier than the interior of the product. It has been reported that the water activity corresponding to maximum rates of browning in potato products is approximately 0.73 (Nursten, 2005). This differs from the data presented for dried milk products (Figure 10.2). It appears that the water activity of instant noodle strands falls very rapidly (Tables 10.1 and 10.2) so that it is expected that the conditions are less than optimal in relation to those likely to promote Maillard reactions (Figure 10.2) and acrylamide formation.

In reviewing the situation in potato strips, Gökmen and Palazoğlu (2008) explained that during frying the heat supplied to the surface of the product by the oil is firstly utilised in increasing the surface temperature to a point just above that at which water boils (103-104°C). After this point, the evaporation of water molecules takes a large proportion of the incoming energy. It was suggested that for lower oil temperatures (<150°C), there is a limitation in the energy input and this effectively prevents the surface temperature reaching above 120°C. However, at relatively high oil temperatures (>170°C), the energy input is sufficient to cause rapid evaporation of the moisture while at the same time increasing the temperature. Therefore within a short duration the formation of acrylamide is strongly favoured.

10.5 The relationship between colour and acrylamide content of instant noodles

The colour of noodles is an especially important parameter influencing sensory responses and consumer perceptions of most styles of Asian noodles including instant noodles. It is well established that most consumers generally accept a uniform, clean white or yellow colour when selecting Asian noodle products. However, to date no studies have demonstrated that there is a direct relationship between colour or the occurrence of browning reactions in noodles and the formation of acrylamide.

It has previously been suggested that, due to the potential for very rapid measurement, colour analysis may be used as an indicator of acrylamide formation in foods. This approach has been studied for French fries and potato products (Mestdagh, Castelein, Van Peteghem, & De Meulenaer, 2008). Gökmen and Senyuva (2006b) found a relationship between the changes of acrylamide content and the redness component (a*) during heating green coffee, wheat flour and potato chips. Thus, the redness parameter a* may be a reliable indicator of acrylamide content in potato chips and green coffee. However, in wheat flour the parameter a* was not found to follow a similar trend to the acrylamide content.

In order to study the potential of colour measurement in instant noodles, experiments were designed to examine the relationship between colour and acrylamide formation. For this, the colour of noodles was investigated at different frying times and temperatures. For the noodle samples prepared for this purpose under controlled laboratory conditions, colour was measured using the Minolta Chroma Meter using the procedure described in Section 6.4.3. Readings were taken after the frying stage in the manufacturing process and the results are shown in Figures 10.4, 10.5, 10.6 and 10.7.

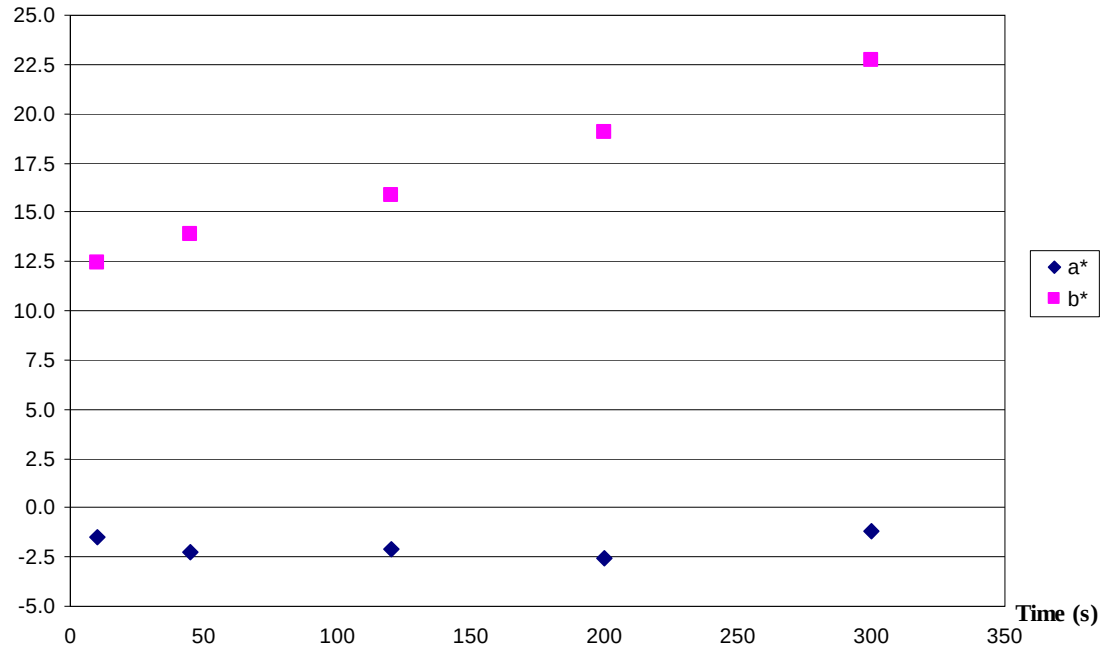


Figure 10.4 Colour changes (measured as a* and b* values) following frying for varying periods of time at a constant temperature of 145 ± 5°C

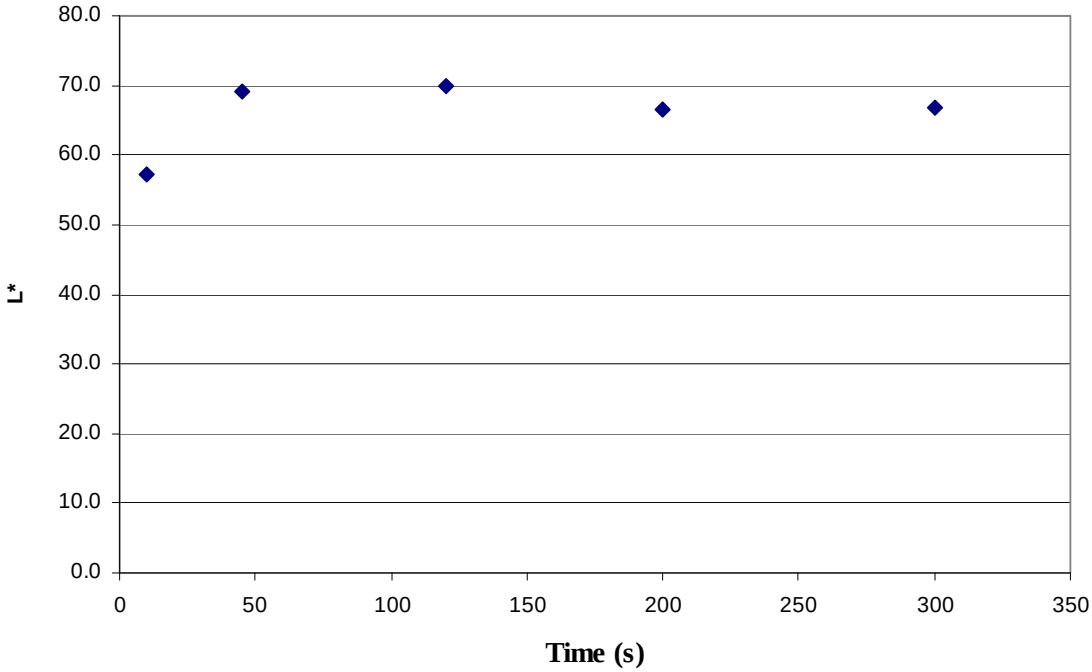


Figure 10.5 Change in the lightness of instant noodles, followed in terms of the parameter L*, during frying at a constant temperature of 145 ± 5°C

From Figure 10.4, the chroma component b* (which effectively ranges from blue when negative to yellow for positive values) shows a significant change during the period of frying while increasing frying time did not show a significant affect in terms of the parameter a* (which measures the balance between green and red appearance). There are significant changes in parameter b*, with instant noodles tending to become more yellow as frying proceeds. However, there was no clear relationship between colour and acrylamide formation. A linear correlation between the b* parameter and time was observed but the a* parameter did not show a similar trend to that of the b* value. It appears that the b* value for instant noodles does not only reflect the occurrence of browning reactions but also is also related to the fat uptake during frying.

Accordingly, a further study was designed to examine any relationship between colour and acrylamide formation at different frying temperatures and the results are presented in Figures 10.6 and 10.7. There appeared to be no clear relationship between the formation of the toxin and any of the colour parameters (a*, b* and L*). In contrast, a linear correlation between acrylamide content and the redness component (a*) was reported in a previous study of potato chips, as the frying temperature increased from

120 to 180°C (Pedreschi, Moyano, Kaack, & Granby, 2005). Potato slices tend to become darker as higher frying temperatures. According to Pedreschi, Kaack, and Granby (2006), the parameter L* and a* showed high correlations with acrylamide content in French fries and this may reflect the observations that acrylamide formation in French fries increased as higher frying temperatures were used.

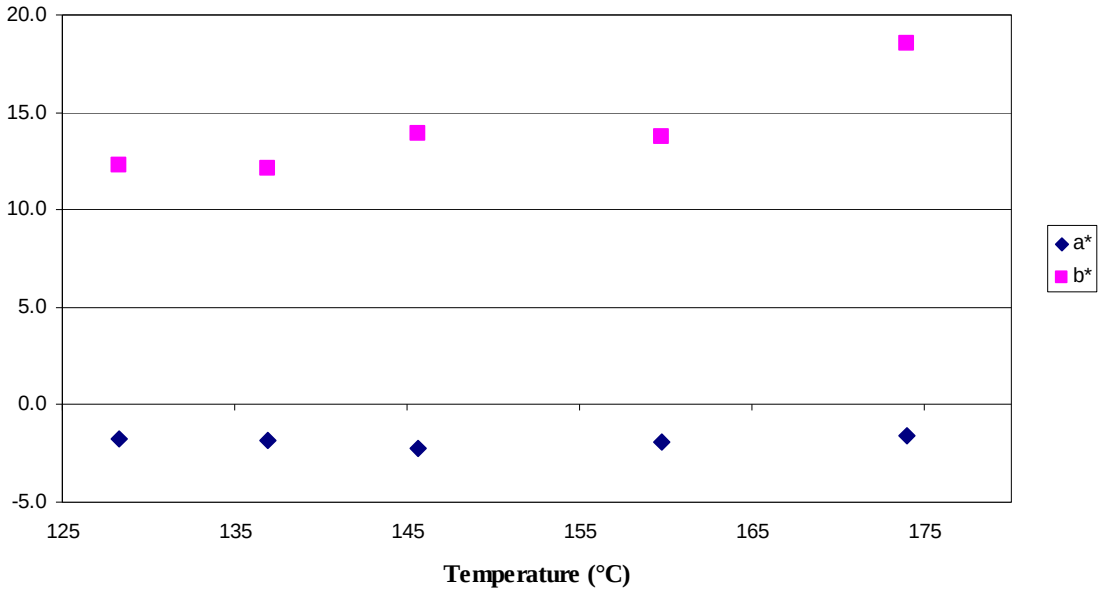


Figure 10.6 Colour changes (measured as a* and b* values) following frying at a constant time of 45 s

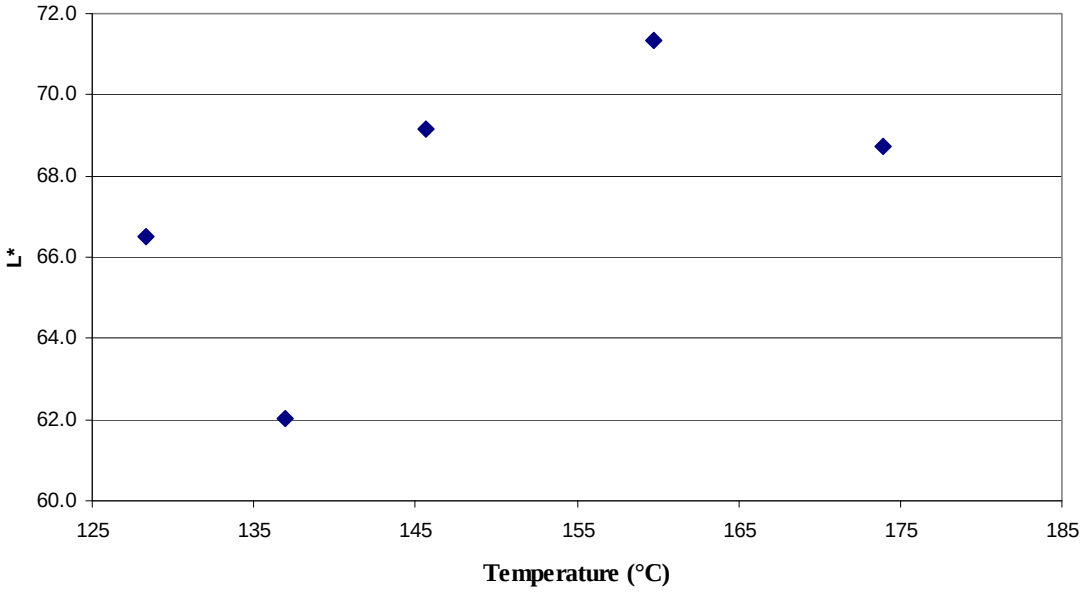


Figure 10.7 Change in the lightness of instant noodles, followed in terms of the parameter L*, during frying at a constant frying of 45 s

In relation to this study, it is concluded that the colour parameters do not provide any indicators which can be effectively used to estimate acrylamide formation in instant noodles. Although prolonged time and higher temperatures of frying result in higher values of the parameter b*, the data presented (Figures 10.4 and 10.6) confirm that acrylamide formation is not linearly related to any of the colour measurements.

10.6 The influence of pH on acrylamide content

In processing of instant noodles, the primary formulation is typically flour, water, salt (NaCl), and alkaline salts. As reviewed in Chapter 4 (Section 4.2.3) the last of these is also referred to as kansui and most commonly a mixture of sodium and potassium carbonates is used (Moss, Miskelly, & Moss, 1986; Miskelly, 1998). The alkaline agents have a number of significant functions including acting to toughen the dough during mixing and sheeting as well as enhancing the strength of the gluten network. They also increase the pH and the higher values may extend the shelf life and inhibit mould growth. Another likely effect is to decrease the activity of the deteriorative enzyme polyphenol oxidase in the dough thereby improving colour and appearance.

The pH of a series of commercial noodles has been measured in the previous phase of this study (Table 9.4) and the pH values ranged from 6.9 to 9.1. For comparison, the pH values obtained for another set of commercial instant noodles and published by Bui and Small (2007c), five brands were measured and the results were between 6.1 and 6.8. All of these are interpreted as being consistent with the incorporation of kansui as alkaline agents, which were declared as ingredients on the labels. It is noted that for white salted noodle products, where the dominant influence on pH is that of the wheat flour, the typical pH values were previously reported to be around 5.5 (Bui and Small, 2007d, 2007e). In the current study, when samples of instant noodles were prepared in the laboratory using the basic formulation, the resultant pH was approximately 7.0.

A series of experiments was set up to investigate the influence of the pH of the instant noodle formulation on acrylamide content. For this, pH values ranging from 6.0 to 11.0 were examined. For all samples kansui was included in the formulation and in order to obtain low pH, the kansui solution, prior to incorporation into the dough, was adjusted

to a pH of approximately 4.0 by addition of 1M HCl. The pH of the resultant noodles was 5.9. Noodles having a more alkaline pH were prepared by increasing the amount of Na_2CO_3 from 0.08% to 0.2% and 0.6% and these resulted in pH readings of 8.5 and 10.7, respectively. For each of the four different noodle samples, the acrylamide content was analysed and the results are presented in Figure 10.8.

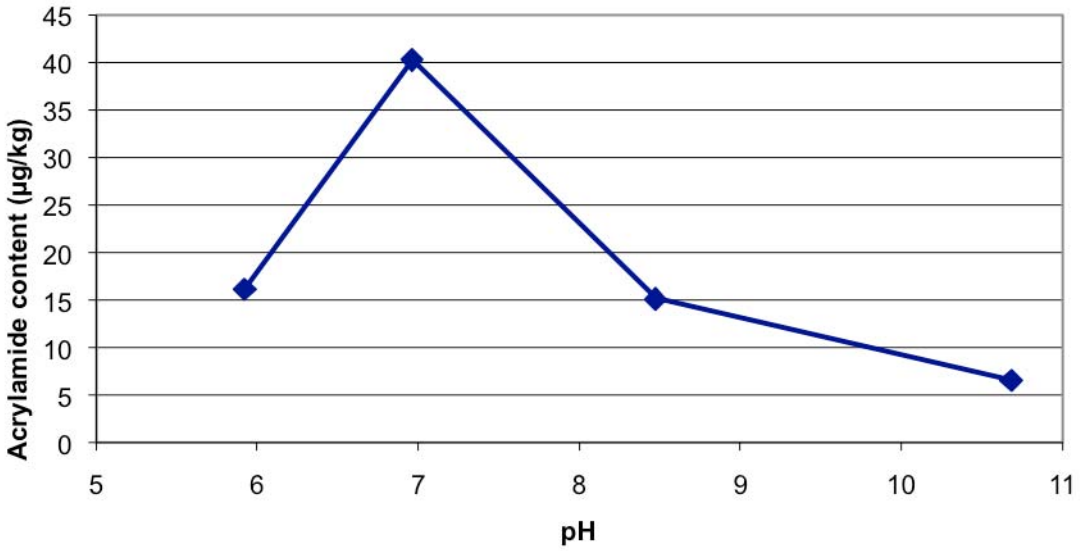


Figure 10.8 Acrylamide content of instant noodles prepared at different pH values (conditions used were frying at 145°C for 45 s)

From Figure 10.8, it was found that the acrylamide content reached a maximum at a pH of approximately 7.0 and then decreased as the pH increased above this. As described in Chapter 3, the influences of pH on acrylamide have previously been examined and the formation of acrylamide is believed to be based upon the Maillard reaction through the formation of the corresponding Schiff base. At low pH, the amino group of the reactant asparagine is likely to be in the fully charged form and therefore it will be unavailable to participate in the reaction.

As well as earlier research into Maillard reactions, there have been specific experiments on acrylamide formation. In a high moisture model system consisting of asparagine and glucose, the effect of pH was studied by heating at 150°C for 30 min in a sealed container. It was found that a decrease of pH from 7.0 to 4.0 resulted in a 99.1% reduction in the acrylamide content (Jung et al., 2003). More recently, in a potato model

system, acrylamide formation reached a peak at pH between 7.0 and 7.5 and declined as the conditions became more alkaline (Mestdagh et al., 2008a). However, Rydberg et al. (2003) found that acrylamide contents were at a maximum when the pH was approximately 8.0 in homogenised potato models. This probably reflects the effect that lowering the pH converts the nucleophilic free non-protonated amino groups of asparagine to the protonated form and this blocks acrylamide formation (De Vleeschouwer, Van der Plancken, Van Loey, & Hendrickx, 2006). For reaction to occur, the amino group of asparagine must be in the non-protonated form and this is required in the first step of acrylamide formation through the Maillard reaction. The extent of protonation of asparagine can be estimated from the acid ionisation constant of the group (the pK_a value) which is 8.8-8.9 (Budavari, 2001). Therefore, asparagine is in the base form when the pH conditions are around 7.0 allowing reaction with electrophiles. The pH of instant noodles is around 7.0 as a result of the incorporation of alkaline salts so that acrylamide formation is normally high due to the basic formulation.

In relation to the impact of higher pH values, asparagine is also expected to have non-protonated amino groups which are required for acrylamide formation. However, from the current experiments it was found that found that higher pH resulted in reduced levels of acrylamide. This can reflect the issue of cations blocking the formation of the Schiff base which is a key intermediate of acrylamide formation. In previous studies of a fructose and aspargine model system, the effect of cations was studied and the results showed that the addition of divalent cations (Ca^{2+}) appeared to completely prevent formation whereas monovalent ions (Na^{+}) were half as effective in preventing acrylamide formation (Gökmen & Şenyuva, 2007a, 2007b).

Although the mechanisms whereby pH influence acrylamide levels are not completely clear, in summary, decreases in acrylamide were noted upon acidification as observed earlier as well as for higher pH. Therefore one of the various strategies available for a manufacturer of instant noodles who is seeking to reduce the amount of the toxin present, is the manipulation of pH.

10.7 The potential contributions of asparaginase on acrylamide formation in instant noodles

Another alternative approach to reduce acrylamide formation is to remove one or more of the precursor compounds. This could especially involve the reaction of the amino acid, L-asparagine and a specific enzyme catalysing this reaction has been reported. The enzyme, L-asparaginase, has been more fully described in Chapter 3 (Ciesarova, Kukurova, & Benesova, 2010) and this has now been approved for use as an approved food processing aid in various countries including Australia (Food Standards ANZ, 2010c).

However, it has been noted that an asparaginase treatment is not necessarily a simple undertaking in a range of food products due to complexity of food matrices and the presence of the food components which may impact on the effectiveness of the enzyme to catalyse the reaction (Kornburst et al., 2009). The objectives in the current investigation have therefore been to investigate the effect and potential of asparaginase as a strategy for reducing the acrylamide formation in instant noodles. In addition, the factors which might be used to manipulate the reaction have been studied as the interactions between asparaginase and processing parameters of instant noodles might be expected to influence the acrylamide content. In the previous phase it was shown that changing of processing conditions including temperature, time of frying and pH strongly influences the formation of acrylamide content. Based upon research into French fries, it has been highlighted that these factors may vary in industrial situations and there can be a lack of control of relevant daily practice (Sanny, Luning, Marcelis, Jinap, & Van Boekel, 2010).

Instant noodles were prepared according to the formulation and procedure described in detail in Chapter 6. In considering what levels might be trialled for this investigation, the dosage of asparaginase has been based upon the provided by the manufacturer documentation. Copies of the relevant material have been provided in Appendix 4 to this thesis. From these a reduction in the acrylamide levels was achieved in a wide range of dough-based products including crisp bread, biscuits, cookies, crackers and snacks. Some of the data provided in the Novozyme application sheet are summarised in Table 10.3.

Based upon this information, for the current trials, three levels of enzyme incorporation were selected for comparison with control (no treatment) formulations. Those chosen were 1000, 2000 and 5000 ASNU/kg flour and, for use in the current experiments, suitable amounts of the enzyme preparation were dissolved in the kansui solution used in the formulation of the noodles. It is noted that considerable care was taken in the choice of approach and the conditions used, reflecting the findings of Amrein, Schönbächler, Escher, and Amadò (2004a): in a study of asparaginase in gingerbread manufacture, these workers found that the technique of enzyme incorporation is crucial and the addition of enzyme in the aqueous solution is more effective than direct mixing of the powder during dough mixing.

Table 10.3 Data provided by the suppliers of the commercial asparaginase preparation showing recommended dosages for selected cereal foods

Food product	Acrylamide content in products (ppb)	Amount of asparaginase added (ppm)	Acrylamide reduction (%)	Recommended dosage (units/kg flour)
Crisp bread	600	150	50	500-2000
Toasted bread	58	300	40	500-1000
Ginger cookies	620	1430	50	1000-2000
Semi-sweet biscuits	360	300	85	500-1000
Crackers	150	60	75	200-1000
Potato chips	5000	600	77	1500-2500
Pretzels	230	70-145	>90	250-500
Cheese cracker	75	145	75	250-100

Note Source of data used was adapted from Novozymes (2010)

For the current trials, the specific conditions chosen for processing involved deep frying at the constant temperature of 145.0 ± 5.0°C for a period of 45 s. A further issue considered in designing the experiments was that for effective reduction of the acrylamide, the enzyme might take some time to act upon the free asparagine found in the dough and originating in the wheat flour. Therefore the usual period of resting of the

dough prior to sheeting (30 min) was used and a further group of samples was allowed to rest for an extended period of 90 min. For analysis, pH values of the doughs and acrylamide content of the instant noodles were measured and the results obtained are shown in Table 10.4 as well as Figures 10.9 and 10.10.

Table 10.4 pH values of doughs at different asparaginase concentrations and resting times

Asparaginase level (ASNU/kg flour)	pH	
	30 min	90 min
0	7.27	7.27
1000	7.20	7.21
2000	7.18	7.20
5000	7.05	7.07

The result for pH values of in Table 10.4 indicates no significant differences for either resting time or asparaginase level. Among the factors that might be expected to affect the activity of the enzyme and also acrylamide formation is the pH conditions within the dough. This partly reflects the impact of pH on the Maillard reaction which is the main pathway of acrylamide formation (Kukurová et al., 2009). However, Table 10.4 shows pH values of the dough at different asparaginase concentration are all very similar at approximately 7.0. This pH is within the optimum range reported for the asparaginase enzyme which is described as being 5.0-8.0 (Novozymes, 2010).

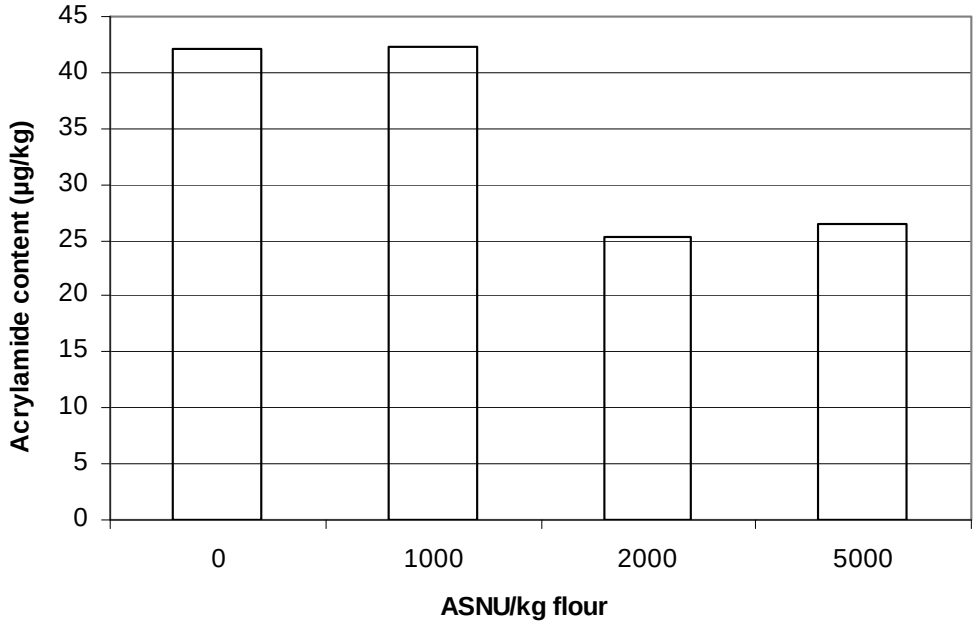


Figure 10.9 Acrylamide content of instant noodles after incubation of the dough for 30 min with three concentrations of L-asparaginase

In the control samples (no enzyme addition), acrylamide levels of 42 µg/kg were measured for 30 min resting times. This value is similar to those found from analyses in previous experiments described in this Chapter. The difference between the measurements of acrylamide for the control samples shown in Figures 10.9 and 10.10 probably reflects some level of experimental variation rather than the difference between resting time as no difference would normally be expected in the acrylamide levels.

A clear and substantial decrease in acrylamide content of instant noodles was observed as a result of enzyme addition but this was dependent upon the level of asparaginase added. The concentrations of 2000 and 5000 ASNU/kg flour showed reductions in the acrylamide content of approximately 39%. However it was consistently observed that the application of asparaginase at concentration as low as 1000 ASNU/kg flour resulted in no significant difference in acrylamide formation compared to the corresponding control treatments. This indicates that in the application of this enzyme it is necessary to ensure that sufficient enzyme is incorporated. In addition, the data indicate significant potential for acrylamide reduction during industrial processing of instant noodles and is

an effective strategy for acrylamide reduction as long as the enzyme concentration is at least 2000 ASNU/kg flour.

In typical commercial production of instant noodles, the dough is rested for 30 min. It was decided that in the current trials, for the effect of addition of asparaginase, a prolonged resting time of 90 min would be investigated. The results are illustrated in Figure 10.10 and this shows that the extended resting time has not obviously reduced acrylamide content compared to those achieved for 30 min resting time (Figure 10.9). These results appear to contrast with those of Hendriksen, Kornbrust, Ostergaard, and Stringer (2009) who investigated semisweet biscuits products. As might be expected, these workers found a greater acrylamide reduction as the resting time was increased. It is possible that apparent differences between the results may be due to the relatively low water addition typically used in the preparation of noodle doughs (35%) compared to values of more than 50% applied in the formulation of many other dough based products. The low moisture conditions in the noodle dough may influence the mobility of the enzyme and the accessibility of the substrate within the dough matrix. Despite the differences between the results for this and the previous study, it is concluded that a practically useful reduction in acrylamide content of around 40% could be achieved during the processing of instant noodles.

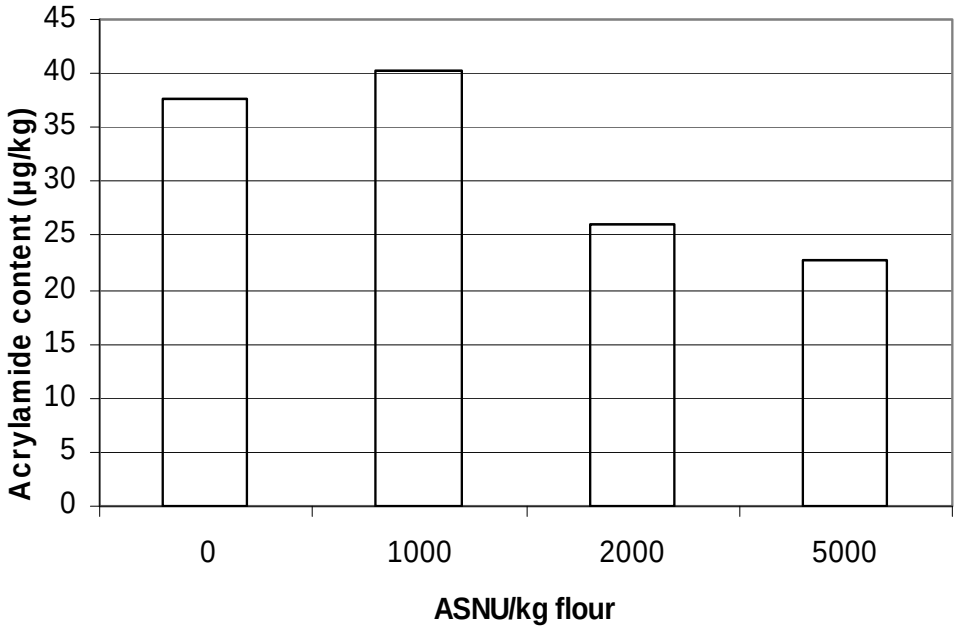


Figure 10.10 Acrylamide content of instant noodles after incubation of the dough for 90 min for three different levels of addition of L-asparaginase

10.8 Summary of results on reducing acrylamide formation

In the current study, the impacts of various factors upon acrylamide contents were studied. These trials were designed so that strategies might be developed in order to minimise acrylamide formation during processing of instant noodles. From these evaluations it was found that:

- Frying temperature and time strongly influence acrylamide formation. In particular, prolonged frying time and higher frying temperatures both resulted in reduced levels of the toxin in the product. The lowest amounts of acrylamide were found for noodles fried at approximately 160°C and for periods of at least 200 s.
- pH value is a particularly important factor: maximum formation of acrylamide occurred at a pH of 7.0. Manipulation of the formulation so that there was either a decrease or increase in the pH of the dough appeared to be effective in minimising acrylamide formation.
- Colour is not a suitable indicator to estimate acrylamide content in instant noodles.

From the investigation of the commercial asparaginase, the addition of this enzyme did appear to reduce the acrylamide content of the instant noodles. Although, the higher levels of incorporation and prolonged resting times did not show significant benefits, a suitable asparaginase level was found to be 2000 ASNU/kg flour, used in conjunction with a resting time of 30 min. At these treatment conditions, the reduction in acrylamide content achieved was approximately 40% and this is a useful additional strategy which could lead to the minimisation of the overall intake of acrylamide by consumers of instant noodles.

Chapter 11

General discussion and conclusions

The purpose of this chapter is to summarise the results obtained during the current study, draw final conclusions and provide recommendations for further studies.

11.1 Introduction

This investigation has focused on instant noodles which are a major food globally and their popularity continues to grow rapidly around the world due to the convenience provided for consumers. These products have the potential to contribute significantly to dietary intakes of essential nutrients as well as that of any undesirable components which may be present. Previously published data indicated that niacin content in wheat flour is relatively low and therefore products made from this are typically not expected to be good dietary sources of niacin. Whilst noodles are high in some important nutrients and are also potentially vehicles for fortification, the recent discovery that acrylamide can form during processing of some foods indicates that instant noodles may contain at least measurable quantities of this toxin. There is increasing evidence that acrylamide can be formed in foods generally during heat treatment due to the Maillard reaction.

The research described in this thesis has investigated niacin and acrylamide in instant Asian noodles and the results have been presented in the following broad areas:

1. Selection of a suitable procedure for analysis of niacin, along with its validation and application for studies of instant noodles;
2. An investigation of the stability of niacin in instant noodles and the potential for fortification;
3. Selection of a suitable procedure for acrylamide and validation for samples of instant noodles;
4. The application of this procedure to the measurement of commercial instant noodles; and

5. A laboratory evaluation, under carefully controlled conditions, of strategies that can be used to minimise the formation of acrylamide in instant noodles.

11.2 Selection and validation of niacin analysis procedure

The purpose of this preliminary work was to select a suitable and reliable procedure for measurement of niacin content of instant noodles. A combination of acid and alkaline extraction followed by SPE was identified as a useful approach. This was investigated and HPLC provided good chromatographic separation. However, the difficulty with niacin analysis was the interference of components in the food matrix that co-eluted during extraction. An SCX column was effectively utilised to remove the interferences before injection to HPLC and the results obtained gave a good recovery of niacin for the AACC reference sample selected for validation purposes.

11.3 The stability of niacin during instant noodle processing

Instant noodles were prepared in the laboratory under controlled conditions and incorporation of niacin in the form of nicotinic acid for fortification purposes was studied. It was found that no significant losses of niacin occurred at any of the steps in the processing of the noodles, even during steaming and frying processes when conditions of elevated temperatures apply. This contrasts with previous research on other B-group vitamins for which significant losses have been observed. Therefore, the high level of stability of nicotinic acid during processing confirms the hypothesis that instant noodles are a suitable vehicle for niacin fortification. No necessity for microencapsulation or other protective strategies are required and these products can be considered for fortification purposes so that they may in future be regarded as good dietary sources of niacin.

11.4 Selection and validation of acrylamide analysis procedure

A thorough evaluation was carried out for methods of acrylamide analysis and involving a variety of published methods. These were based upon GC coupled with various detectors including FID, MS as well as NPD and ECD. Only the last of these options was found to provide a good level of sensitivity and suitable separation patterns were achieved with ECD. In considering alternative extraction procedures, those incorporating a derivatisation step were found to give good selectivity for application to

extracts of noodle samples when used in conjunction with ECD. The results showed that bromination with saturated bromine water gave reasonably reliable results when validation studies were carried out.

11.5 Analysis of acrylamide in instant noodle samples

A series of instant noodle samples were analysed for acrylamide content using the extraction and analysis methods validated from the previous phase. The samples were selected to reflect some of the variability known to exist amongst different brands of noodles. Among the aspects considered in the choice of samples were variations in the ingredients declared on the labels, the textural attributes as well as the colour characteristics. Samples were specifically selected so that as many different countries of origin were represented as possible. In these preliminary studies, it was found that the contents of acrylamide in the instant noodles ranged from 11.6 to 77.4 μg/kg. From the results of commercial samples, it was difficult to draw conclusions about the factors that might have been related to acrylamide content. In particular the product pH values did not show any direct relationship to the amount of acrylamide measured. Therefore the results indicate that a combination of a number of factors may be relevant to the prediction of acrylamide formation.

11.6 The influence of processing parameters on acrylamide formation in noodles prepared in the laboratory

Accordingly instant noodles were prepared in the laboratory under controlled conditions. The purpose of this phase of the work has been to investigate the effects of processing conditions on the acrylamide content which can lead to the formulation of strategies for reducing acrylamide contents of instant noodles. In order to assess the factors which might influence the formation of acrylamide, a series of instant noodle samples were prepared. Frying time and temperature were evaluated and the data demonstrated that frying for more than 150 s at 145°C can effectively reduce acrylamide content by approximately 57%. The acrylamide content was also strongly related to the pH, with maximum levels being found in samples having a pH of 7.0. Therefore to control acrylamide content, decreased or increased of pH is another approach that might be used to acrylamide formation. On the other hand, it was also found that the colour of the noodles is not a reliable indicator to predict acrylamide

content. Moreover, the application of asparaginase is effective to reduce levels of acrylamide in the final products. A suitable asparaginase level is 2000 ASNU/kg flour when used with a 30 min resting time following dough mixing. The resulting reduction in acrylamide levels corresponded to 40% of that present in the control treatments.

11.7 Possible areas for future research

The current study has been focused specifically on instant Asian noodles. Whilst providing specific conclusions related to niacin and acrylamide, there are a range of areas which were beyond the scope of this project and these warrant further study. The purpose here is to briefly recommend particular topics for ongoing research.

Procedures have been developed, evaluated and validated, for extraction and analysis of both niacin and acrylamide. The challenges presented by these phases of the study reconfirm the importance of evaluation and adaptation of existing methods when these are being applied to particular foods. The recent research on thiamin, riboflavin, vitamin B6, folates and folic acid described in earlier chapters of this thesis has demonstrated the significance of this principle and the necessity of validation. In particular, the extraction of minor components from a specific food matrix and the minimisation of matrix effects in chromatographic analyses requires close attention.

The procedures established in the current study may therefore form the basis for evaluating methods for other cereal grain foods. It is recommended that further studies be carried out using these approaches to extend our understanding of niacin and acrylamide in other grain based foods.

If, in the longer term, it became necessary to carry out routine monitoring of instant noodles, or other foods having a similar matrix, it would be desirable to develop faster, more cost effective procedures. Based upon the options trialled in the current study, the use of microfiber absorption provided a relatively convenient preparation of extracts. The limitation that would have to be overcome relates to the robustness of the fibre components that are available at this time.

In relation to the potential fortification of Asian noodles, including instant products, a number of vitamins and other components remain to be investigated. These include pantothenic acid as well as the fat-soluble vitamins. Much remains to be done if instant noodles are to contribute more broadly to the nutrition and wellbeing of the increasing number of consumers who enjoy these products.

In relation to acrylamide, the current study provides a number of alternatives which might be considered by commercial processors of instant noodles. Further work would be required to develop a combination of strategies that is both effective in reducing the levels of acrylamide whilst maintaining the sensory attributes which are attractive to consumers. For example, it would be appear to be quite simple for a manufacturer to adjust the ingredient formulation so that the pH of the product was below 6.5. This would reduce acrylamide contents substantially as well as effecting enhanced retention of a number of vitamins including thiamin and riboflavin. However the lower pH would also impact upon starch gelation and hence product texture. It is recommended that further research consider the interactions of processing variables on each of these factors. In addition, the use of semi-commercial scale trials may be useful if such facilities could be accessed.

Finally this project has sought to investigate instant Asian noodles as a safe product, supplying essential nutrients and contributing to health and wellbeing. It is hoped that this work will provide a basis for ongoing research in this important area so that wheat flour may continue to benefit and satisfy an ever expanding global population.

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Note In this thesis referencing has followed the recommendations of the American Psychological Association (APA, 2010)

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Appendix 1

Details of AACC Standard Reference Sample used in the establishment and validation of niacin analysis. The following tabulation was supplied with the AACC reference sample used in the current study.

AACC Standard Reference Sample VMA 406*

Assay	Mean**	No. of Analyses	Std. Dev.	C.V. (%)	Range
Moisture, %	3.89	27	0.29	7.5	3.16-4.33
Fat, % (Ether extract after Acid Hydrolysis)	3.01	17	0.69	22.9	1.5-4.37
Fat, % (Ether Extract)	1.31	15	0.15	11.5	1.01-1.61
Ash, %	2.188	23	0.051	2.3	2.05-2.27
Protein, % (N x 6.25)	4.38	21	0.18	4.1	3.88-4.72
Vitamin A, IU/100g	1435.2***	17	710	49.5	109-2372
Vitamin C, mg/100g	16	19	6.59	41.2	0-24.9
Thiamine, mg/100g	1.31	11	0.21	16.0	.98-1.72
Riboflavin, mg/100g	2.08	13	0.23	11.1	1.6-2.46
Niacin, mg/100g	26.51	8	3.73	14.1	20.93-32.85
Vitamin B6, mg/100g	1.84	5	0.15	8.2	1.6-1.99
Vitamin B12, μg/100g	8.84	4	1.08	12.2	8.1-10.4
Folic Acid, mg/100g	0.723***	6	0.506	70.0	0-1.13
Pantothenic Acid, mg/100g	0.26	4	0.1	38.5	.13-.37
Iron, mg/100g	16.8	21	1.4	8.3	13.1-19.3
Calcium, mg/100g	284.9	20	18	6.3	241.1-323.0
Magnesium, mg/100g	30.1	17	2.4	8.0	26.3-35.9
Zinc, mg/100g	19.6	19	2	10.2	15.7-24.4
Copper, mg/100g	0.103***	15	0.077	74.8	0-0.28
Phosphorus, mg/100g	202.9	16	9.7	4.8	183-217
Potassium, mg/100g	102.4	17	11.7	11.4	84.3-128
Manganese, mg/100g	0.57	15	0.04	7.0	.49-.64
Sodium, g/100g	0.454	20	0.04	8.8	.367-.533

*VMA 406 is a ground-up fortified ready-to-eat cereal.

Means are calculated ***after* deleting the outlier values according to:
Grubbs, F.E. 1969 Technometrics 11(1): 4, Table 1.

*** These data do not meet our criteria for an analytical reference standard, but they are included here for what they might be worth to the users of this sample.

Appendix 2

Details of NIST Standard Reference Sample used in the establishment and validation of acrylamide analysis. The following tabulation was supplied with the NIST reference sample used in the current study. Note that only sections related to acrylamide are included here.

National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 2387

Peanut Butter

This Standard Reference Material (SRM) is intended primarily for use in validating methods for determining proximates, fatty acids, calories, vitamins, elements, amino acids, aflatoxins, and acrylamide in peanut butter and similar matrices. This SRM can also be used for quality assurance when assigning values to in-house control materials. A unit of SRM 2387 consists of three jars of peanut butter containing 170 g each.

Certified Concentration Values: The certified concentration values of fat, selected fatty acids, elements, and tocopherols in SRM 2387 are provided in Tables 1 and 2. Values were derived from the combination of results provided by NIST and collaborating laboratories. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or accounted for by NIST [1]. The certified values in this material are the equally weighted means of the mean NIST result and the mean of the measurements made by collaborating laboratories; the associated uncertainties are expanded uncertainties at the 95 % level of confidence [2,3]. Values are reported on an as-received (not dry-mass) basis in mass fraction units [4].

Reference Concentration Values: Reference concentration values for additional proximates, fatty acids, amino acids, calories, total dietary fiber, vitamins, aflatoxins, and acrylamide are provided in Tables 3 through 7. Reference values are noncertified values that are the best estimate of the true values based on available data; however, the values do not meet the NIST criteria for certification [1] and are provided with associated uncertainties that may reflect only measurement reproducibility, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods.

Expiration of Value Assignment: The value assignment of this SRM is valid until **31 December 2009**, within the measurement uncertainties specified, provided the SRM is handled and stored in accordance with the instructions given in this certificate. Value assignment is nullified if the SRM is damaged, contaminated, or modified.

Maintenance of SRM Value Assignment: NIST will monitor this SRM over the period of its value assignment. If substantive technical changes occur that affect the value assignment before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet) will facilitate notification.

Coordination of the technical measurements leading to the certification of this SRM was performed by K.E. Sharpless of the NIST Analytical Chemistry Division and H.B. Chin, I-P. Ho, and D.W. Howell of the National Food Processors Association (NFPA, Dublin, CA and Washington, DC).

Analytical measurements at NIST were performed by C.S. Phinney, K.E. Sharpless, and L.J. Wood of the NIST Analytical Chemistry Division. Analyses for value assignment were also performed by the laboratories listed in Appendices A through C.

Statistical analysis was provided by J.H. Yen of the NIST Statistical Engineering Division.

Stephen A. Wise, Chief
Analytical Chemistry Division

Robert L. Watters, Jr., Chief
Measurement Services Division

Gaithersburg, MD 20899
Certificate Issue Date: 12 January 2007
See Certificate Revision History on Page 8

The support aspects involved in the issuance of this SRM were coordinated through the NIST Measurement Services Division.

NOTICE AND WARNING TO USERS

Storage: The peanut butter should be frozen at –20 °C until required for use

WARNING: For laboratory use only. **NOT** for human consumption

INSTRUCTIONS FOR USE

Prior to removal of a test portion for analysis, a jar of peanut butter should be thawed under refrigeration overnight. The contents of a jar should be mixed thoroughly prior to removal of a test portion. Test portions used for NIST analyses described below were: 5 g to 7 g for tocopherols; 1 g for fat and fatty acids; and 0.5 g for elements.

PREPARATION AND ANALYSIS

Preparation: SRM 2387 is creamy peanut butter containing roasted peanuts, sugar, partially hydrogenated vegetable oils (48 % rapeseed, 40 % cottonseed, and 12 % soybean oil), and salt, and was prepared for NIST as part of a larger production run. Raw, shelled Florunner (primarily) peanuts were received from several suppliers and were roasted. The skins were removed from the roasted peanuts, and discolored peanuts were discarded. The roasted peanuts were then ground, and the remaining ingredients were added. After mixing, the peanut butter was further ground to a fine particle size, air was removed, and the peanut butter was cooled and packed in colorless polyethyl tetraethylene (PETE) jars with white screw caps and foil liners.

NIST Analyses for Fat: One set of three samples of peanut butter was prepared for gravimetric analysis of fat. One-gram portions of peanut butter were mixed with diatomaceous earth. The mixture was then briefly chilled at 4 °C to improve handling. The fat was then extracted from the mixture by pressurized fluid extraction (PFE) using hexane:acetone (4:1 volume fraction). Extracts were evaporated under nitrogen and then dried at 100 °C to constant mass.

NIST Analyses for Fatty Acids: Twelve fatty acids were measured in two sets of six samples of peanut butter prepared on two different days. The fat was extracted from approximately 1 g samples of peanut butter by PFE using a mixture of hexane:acetone (4:1 volume fraction). Methyl nonadecanoate (C19:0 fatty acid methyl ester [FAME]) was used as an internal standard. A two-step process employing methanolic sodium hydroxide and boron trifluoride was used to convert the fatty acids to their methyl esters. FAMEs were extracted into hexane and analyzed by gas chromatography with flame ionization detection.

NIST Analyses for Elements: Calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc were measured in eight jars of peanut butter. Two 0.5 g portions were taken from each jar and digested in a nitric, perchloric, and hydrofluoric acid mixture. Because of the high fat content, the samples were predigested on a hotplate before digestion in a microwave oven. Digests were transferred to plastic bottles and diluted with the appropriate volume of 1.5 % (volume fraction) nitric acid. To correct for matrix effects caused by differences between samples and calibrants, the method of standard additions was used; spikes were added to one aliquot prepared from each 0.5 g test portion. Four measurements using inductively coupled plasma optical emission spectrometry (ICP-OES) were made and averaged for each sample and each spiked solution. Results were corrected for spike recoveries.

NIST Analyses for Tocopherols: δ-Tocopherol, γ- (plus β-) tocopherol, and α-tocopherol were measured in test portions taken from six jars of peanut butter over a seven-day period. (The peanut butter may contain β-tocopherol, but the chromatographic system described below is incapable of resolving β- and γ-tocopherol; the instrument was calibrated using only γ-tocopherol.) Samples of approximately 5 g to 7 g were saponified using potassium hydroxide. Analytes were extracted into a mixture of diethyl ether and hexane, which was subsequently evaporated, and the analytes were redissolved in a mixture of ethanol and ethyl acetate. Samples were analyzed by liquid chromatography (LC) on a C_{18} column; analytes were eluted using a gradient of acetonitrile, methanol, and ethyl acetate [5]. A programmable UV/visible absorbance detector set to 450 nm for measurement of *trans*-β-apo-10'-carotenal oxime

(the internal standard) and a fluorescence detector (excitation wavelength of 295 nm, emission wavelength of 335 nm) were used for quantitation of the tocopherols.

Analyses by Collaborating Laboratories: Data from three additional sources were used for certification of this material: an interlaboratory comparison exercise organized by the NFPA Food Industry Analytical Chemists Subcommittee (FIACS) with 13 laboratories participating (Appendix A); four laboratories participating in an exercise in which only aflatoxins were measured (Appendix B); 15 laboratories participating in an exercise organized by the Joint Institute for Food Safety and Applied Nutrition (JIFSAN) Acrylamide Working Group in which acrylamide was measured (Appendix C). Not every laboratory measured every analyte. The laboratories listed in Appendix A were asked to use AOAC methods or their equivalent, to make single measurements from each of two jars, and to report the analytical method that was used. The laboratories listed in Appendix B were asked to use their usual methods to make single measurements of aflatoxins in each of three jars. The laboratories listed in Appendix C were asked to use their usual methods to make duplicate measurements of acrylamide in a single jar. A summary of the methodological information and the number of laboratories using a particular analytical technique is provided in Appendix D. The methods used by NIST are included in this listing as well.

Homogeneity Assessment: The homogeneity of calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, zinc, fatty acids, and tocopherols was assessed at NIST using the methods described above. A small but statistically significant heterogeneity was found for some analytes, and an inhomogeneity component of approximately 1 % has been included in the expanded uncertainty for all analytes.

Value Assignment: The laboratories listed in Appendix A reported values for 2 to 12 analyses. The laboratories listed in Appendix B reported values for three to nine analyses. The laboratories listed in Appendix C reported values for two to three analyses. The mean for each laboratory was determined from these values, and a mean of laboratory means was calculated. In cases where NIST also made measurements, this mean of means was averaged with the NIST mean to obtain the assigned value. In cases where NIST did not make measurements, the mean of laboratory means became the assigned value.

Table 1. Certified Concentration Values for Fat and Selected Fatty Acids[a]

	Mass Fraction (%)
Fat (Extractable)	51.6 ± 1.4
Fat (Sum of Fatty Acids)[b]	49.8 ± 1.9
Saturated Fat[c]	10.4 ± 0.2
Monounsaturated Fat[c]	24.4 ± 0.9
Polyunsaturated Fat[c]	13.2 ± 0.4

	Mass Fraction (%) as the Triglyceride	Mass Fraction (%) as the Fatty Acid
Tetradecanoic Acid (C14:0) (Myristic Acid)	0.025 ± 0.002	0.024 ± 0.002
Hexadecanoic Acid (C16:0) (Palmitic Acid)	5.18 ± 0.15	4.94 ± 0.15
(Z)-9-Hexadecenoic Acid (C16:1 n-7) (Palmitoleic Acid)	0.046 ± 0.011	0.044 ± 0.010
Octadecanoic Acid (C18:0) (Stearic Acid)	2.23 ± 0.08	2.13 ± 0.08
(Z)-9-Octadecenoic Acid (C18:1 n-9) (Oleic Acid)	24.43 ± 0.94	23.38 ± 0.90
(Z)-11-Octadecenoic Acid (C18:1 n-7) (Vaccenic Acid)	0.266 ± 0.017	0.255 ± 0.016
(Z,Z)-9,12-Octadecadienoic Acid (C18:2 n-6) (Linoleic Acid)	13.75 ± 0.43	13.15 ± 0.41
(Z,Z,Z)-9,12,15-Octadecatrienoic Acid (C18:3 n-3) (Linolenic Acid)	0.031 ± 0.001	0.030 ± 0.001
Eicosanoic Acid (C20:0) (Arachidic Acid)	0.739 ± 0.030	0.710 ± 0.029
(Z)-11-Eicosenoic Acid (C20:1 n-9) (Gondoic Acid)	0.669 ± 0.032	0.643 ± 0.031
Docosanoic Acid (C22:0) (Behenic Acid)	1.88 ± 0.08	1.81 ± 0.08
Tetracosanoic Acid (C24:0) (Lignoceric Acid)	0.808 ± 0.045	0.781 ± 0.044

[a] Each certified concentration value for fat and individual fatty acids, expressed as a mass fraction on an as-received basis, is the weighted mean of results provided by the laboratories listed in Appendix A and NIST. The uncertainty in the certified values, calculated according to the method described in the ISO and NIST Guides [2,3], is expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-laboratory, within-laboratory, and inhomogeneity components of uncertainty. The coverage factor, k, is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and 95 % confidence for each analyte. Analytical methodology information is provided in Appendix D. Some fatty acid data from collaborating laboratories were excluded if the laboratory's mean result was more than three standard errors from the mean of the interlaboratory comparison exercise.

[b] Fat as the sum of the fatty acids represents the sum of quantified individual fatty acid peaks (for which both certified and reference values are provided) as the triglycerides.

[c] The certified values for saturated, monounsaturated, and polyunsaturated fats are sums of the assigned values (certified and reference) for the individual fatty acids (as the fatty acids) in each group. The uncertainty is expressed as an expanded uncertainty, U, at the 95 % level of confidence, and is calculated according to the ISO Guide. The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the measurement error. The coverage factor, k, is determined from the Student's t-distribution corresponding to the appropriated associated degrees of freedom and 95 % confidence.

Table 2. Certified Concentration Values for Elements and Tocopherols[a]

	Mass Fraction (mg/kg)		
Calcium	411	±	18
Copper	4.93	±	0.15
Iron	16.4	±	0.8
Magnesium	1680	±	70
Manganese	16.0	±	0.6
Phosphorus	3378	±	92
Potassium	6070	±	200
Sodium	4890	±	140
Zinc	26.3	±	1.1
δ-Tocopherol	10	±	3
γ- + β-Tocopherol	100	±	19
α-Tocopherol	108	±	11

[a] Each certified concentration value, expressed as a mass fraction on an as-received basis, is the weighted mean of results provided by the laboratories listed in Appendix A and NIST. The uncertainty in the certified values, calculated according to the method described in the ISO and NIST Guides [2,3], is expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-laboratory, within-laboratory, and inhomogeneity components of uncertainty. The coverage factor, k, is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and 95 % confidence for each analyte. Analytical methodology information is provided in Appendix D.

Table 3. Reference Concentration Values for Proximates and Caloric Content[a]

	Mass Fraction (%)		
Solids	99.2	±	2.1
Ash	3.10	±	0.10
Protein	22.2	±	0.5
Carbohydrate (by difference)[b]	25.0	±	1.8
Total Dietary Fiber	5.57		0.42
	Calories		
Caloric Content[c]	629 kcal/100 g	±	15 kcal/100 g

[a] Each reference concentration value, expressed as a mass fraction on an as-received basis, is the weighted mean of results provided by the laboratories listed in Appendix A. The uncertainty in the reference values, calculated according to the method described in the ISO and NIST Guides [2], is expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-laboratory, within-laboratory , and inhomogeneity components of uncertainty. The coverage factor, k, is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and 95 % confidence for each analyte. Analytical methodology information is provided in Appendix D. (The certified values for fat are provided in Table 1.)

[b] Based on fat as the sum of the fatty acids.

[c] The value for caloric content is the mean of individual caloric calculations from the laboratories listed in Appendix A. If the proximate values above are used for calculation, with caloric equivalents of 9, 4, and 4 for fat (as the sum of the fatty acids) protein, and carbohydrate, respectively, the mean caloric content is 637 kcal/100 g.

Table 4. Reference Concentration Values for Fatty Acids[a]

	Mass Fraction (%) as the Triglyceride	Mass Fraction (%) as the Fatty Acid
Heptadecanoic Acid (C17:0) (Margaric Acid)	0.050 ± 0.001	0.048 ± 0.001
Heptadecenoic Acid (C17:1)	0.035 ± 0.006	0.033 ± 0.006
Eicosadienoic Acid (C20:2)	0.017 ± 0.007	0.016 ± 0.007
(Z,Z,Z,Z)-5,8,11,14-Eicosatetraenoic Acid (C20:4 n-6) (Arachidonic Acid)	0.025 ± 0.016	0.024 ± 0.015
(Z)-13-Docosenoic Acid (C22:1 n-9) (Erucic Acid)	0.056 ± 0.012	0.054 ± 0.012

[a] Each reference concentration value, expressed as a mass fraction on an as-received basis, is the weighted mean of results provided by the laboratories listed in Appendix A. The uncertainty in the reference values, calculated according to the method described in the ISO and NIST Guides [2] is expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-laboratory, within-laboratory, and inhomogeneity components of uncertainty. The coverage factor, k is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and 95 % confidence for each analyte. Analytical methodology information is provided in Appendix D.

Table 5. Reference Concentration Values for Amino Acids[a]

	Mass Fraction (%)
Alanine	0.93 ± 0.10
Arginine	2.65 ± 0.31
Aspartic Acid	2.83 ± 0.19
Cystine	0.27 ± 0.01
Glutamic Acid	4.69 ± 0.26
Glycine	1.41 ± 0.12
Histidine	0.55 ± 0.06
Isoleucine	0.77 ± 0.07
Leucine	1.56 ± 0.09
Lysine	0.78 ± 0.08
Methionine	0.21 ± 0.04
Phenylalanine	1.21 ± 0.08
Proline	0.96 ± 0.08
Serine	1.16 ± 0.09
Threonine	0.54 ± 0.08
Tryptophan	0.21 ± 0.06
Tyrosine	0.81 ± 0.14
Valine	0.94 ± 0.09

[a] Each reference concentration value, expressed as a mass fraction on an as-received basis, is the weighted mean of results provided by the laboratories listed in Appendix A. The uncertainty in the reference values, calculated according to the method described in the ISO and NIST Guides [2], is expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent at the level of one standard deviation, the combined effect of between-laboratory, within-laboratory, and inhomomgeneity components of uncertainty. The coverage factor, k, is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and 95 % confidence for each analyte. Analytical methodology information is provided in Appendix D.

Table 6. Reference Concentration Values for Selected Water-Soluble Vitamins[a]

	Mass Fraction (mg/kg)		
Niacin	142	±	6
Pantothenic Acid	10 8	±	3 2
Vitamin B_1 Hydrochloride	0 84	±	0 17
Vitamin B_6	4 66	±	0 62

[a] Each reference concentration value, expressed as a mass fraction on an as-received basis, is the weighted mean of results provided by the laboratories listed in Appendix A. The uncertainty in the reference values, calculated according to the method described in the ISO and NIST Guides [2], is expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-laboratory, within-laboratory, and inhomogeneity components of uncertainty. The coverage factor, k, is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and 95 % confidence for each analyte. Analytical methodology information is provided in Appendix D.

Table 7. Reference Concentration Values for Aflatoxins and Acrylamide[a]

	Mass Fraction (ng/g)		
Aflatoxin B1	4.2	±	0 9
Aflatoxin B2	0.7	±	0 3
Total Aflatoxins[b]	5.0	±	0 5
Acrylamide[c]	87.0	±	7 8

[a] Each reference concentration value, expressed as a mass fraction on an as-received basis, is the weighted mean of results provided by the laboratories listed in Appendices A and B. The uncertainty in the reference values, calculated according to the method described in the ISO and NIST Guides [2], is expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U = ku_c$ where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-laboratory, within-laboratory, and inhomogeneity components of uncertainty. The coverage factor, k, is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and 95 % confidence for each analyte. Analytical methodology information is provided in Appendix D.

[b] The reference value for total aflatoxins is the mean of the laboratory means of the sum of aflatoxins B1 and B2.

[c] Each reference concentration value, expressed as a mass fraction on an as-received basis, is the weighted mean of results provided by the laboratories listed in Appendices A and C. The uncertainty in the reference values, calculated according to the method described in the ISO and NIST Guides [2], is expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-laboratory, within-laboratory, and inhomogeneity components of uncertainty. The coverage factor, k, is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and 95 % confidence for each analyte. Analytical methodology information is provided in Appendix D.

Appendix 3

Minitab output for the One-way ANOVA: niacin content versus processing steps

```
Welcome to Minitab, press F1 for help

One-way ANOVA: niacin versus process

Source   DF      SS     MS      F      P
process   3    7.42   2.47   0 53  0.686
Error     4   18.72   4.68
Total     7   26.15

S = 2 164   R-Sq = 28.39%   R-Sq(adj) = 0.00%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  -----+---------+---------+---------+----
1      2  29.195  0.841          (-------------*-------------)
2      2  26.860  2.942  (--------------*-------------)
3      2  27.705  2.878     (--------------*--------------)
4      2  26.815  1.039  (--------------*--------------)
                          -----+---------+---------+---------+----
                            24.0      27.0      30.0      33.0

Pooled StDev = 2 164


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of process

Individual confidence level = 98 48%


process = 1 subtracted from:

process    Lower  Center  Upper  ---------+---------+---------+---------+
2        -11.147  -2 335  6.477  (--------------*--------------)
3        -10 302  -1 490  7 322    (--------------*--------------)
4        -11 192  -2.380  6.432  (--------------*--------------)
                                 ---------+---------+---------+---------+
                                       -6.0       0.0       6 0      12 0


process = 2 subtracted from:

process   Lower  Center  Upper  ---------+---------+---------+---------+
3        -7.967   0.845  9 657        (--------------*--------------)
4        -8.857  -0.045  8.767     (--------------*--------------)
                                 ---------+---------+---------+---------+
                                       -6.0       0 0       6.0      12 0


process = 3 subtracted from:

process   Lower  Center  Upper  ---------+---------+---------+---------+
4        -9.702  -0.890  7.922     (--------------*--------------)
                                 ---------+---------+---------+---------+
                                       -6 0       0 0       6.0      12 0
```

Appendix 4

Copy of the specification sheet for the Acrylaway® provided by Novozymes

Acrylaway® 3500 BG

Valid from	**2010-03-23**
Product Characteristics:	
Declared enzyme	Asparaginase
Declared activity	3500 ASNU/g
Colour	Off-white Colour can vary from batch to batch. Colour intensity is not an indication of enzyme activity
Physical form	Granulate
Properties	Freeflowing, low-dusting
Approximate density (g/ml)	0.67
Diluents	Wheat flour
Stabilisers	Sodium chloride
	This product is standardized by Documented Addition in a process controlled by Novozymes ISO 9001 quality system. See Documented Addition Info Sheet for further information.
Solubility	Active component is readily soluble in water at all concentrations that occur in normal usage. Standardisation components can cause turbidity in solution.
Production organism	Aspergillus oryzae
Production method	Produced by fermentation of a microorganism which is selfcloned according to the EU definition (further information available upon request). The enzyme protein is separated and purified from the production organism

1 / 2

Product Specification:

	Lower Limit	Upper Limit	Unit
Asparaginase ASNU	3500		/g
Total Viable Count	-	50000	/g
Coliform Bacteria	-	30	/g
Enteropathogenic E.Coli	Not Detected		/25 g
Salmonella	Not Detected		/25 g

The product complies with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemical Codex (FCC).

Packaging: See the standard packaging list for more information.

Recommended Storage:

Best before	When stored as recommended, the product is best used within 12 months from date of delivery.
Storage at customer's warehouse	0-25°C (32°F-77°F)
Storage Conditions	In unbroken packaging - dry and protected from the sun. The product has been formulated for optimal stability. Extended storage or adverse conditions such as higher temperature or higher humidity may lead to a higher dosage requirement

Safety and handling precautions

Enzymes are proteins. Inhalation of dust or aerosols may induce sensitization and may cause allergic reactions in sensitized individuals Some enzymes may irritate the skin, eyes and mucous membranes upon prolonged contact. This product has been developed to resist light mechanical effects However,excessive mechanical wear and tear or crushing may create dust. All spills, however minor, should be removed immediately. Use respiratory protection. Major spills should be carefully shovelled into plastic-lined containers. Minor spills and the remains of major spills should be removed by vacuum cleaning or flushing with water (avoid splashing). Vacuum cleaners and central vacuum systems should be equipped with HEPA filters. Wear suitable protective clothing, gloves and eye/face protection as prescribed on the warning label. Wash contaminated clothes A Material Safety Data Sheet is supplied with all products. See the Safety Manual for further information regarding how to handle the product safely.

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